

***Remarks***

***I. Status of the Claims***

Reconsideration of this Application is respectfully requested.

Upon entry of the foregoing amendment, claims 1-13, 17, 19-32 and 45-70 are pending in the present application, with claims 1 and 45 being the independent claims. Claims 14-16, 18 and 33-44 are sought to be cancelled without prejudice to or disclaimer of the subject matter therein. Claims 1, 3-13, 17, 19-22, 24-27 and 29-32 are sought to be amended. Furthermore, new claims 45-70 are sought to be added. Support for the amendment to claim 1 may be found in the specification as filed, *e.g.*, at page 10, lines 20-23; page 11, lines 20-30, in particular lines 25-28; in Example 1 at page 42 lines 23-26; and in Examples 3 and 4 at page 43, line 29 and at page 44, line 15. The amendments to claims 2-13, 17, 19-22, 24-27 and 29-32 are to improve clarity and remove multiple dependencies from the claims.

Support for new claim 45 may be found in the specification at, for example, page 10, line 32 to page 11, line 2 and 22-30, in particular lines 23 and 24, original claim 1, in Example 1 at page 42, lines 23-26 and Example 2 at page 43, line 14. New claims 46-70 are based on or correspond to original claims 2-6, 14-17 and 19-32.

The amendments to the claims and the newly added claims are believed to introduce no new matter, and their entry is respectfully requested.

Based on the above amendment and the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

***II. Summary of the Office Action***

In the Office Action dated December 11, 2008, the Examiner has made one objection to claims 4-33 and six rejections of claims 1-33. Applicants respectfully offer the following remarks concerning each of these elements of the Office Action. In this respect, for the sake of conciseness, Applicants' remarks are made in relation to original claims 1-33 and amended claims 1-32 presented herewith, respectively. However, those remarks similarly apply to new claims 45-70.

***III. Objection to Claims 4-33***

At page 3 of the Office Action, the Examiner has objected to claims 4-33 under 37 CFR 1.75(c) as being in improper form because a multiple dependent claim cannot depend from any other multiple dependent claim. Applicants have amended claims 4-33 to remove the multiple dependency in the claims. Thus, Applicants respectfully submit that this objection has been rendered moot and request that the claims be further treated on the merits.

***IV. Rejections Under 35 U.S.C. § 112, Second Paragraph***

At pages 4-6 of the Office Action, the Examiner has rejected claims 1-33 under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention. Applicants respectfully traverse these rejections.

***A. Rejection of Claim 1 and Claims 2-33***

At page 4 of the Office Action, section 5, 2nd paragraph, the Examiner has rejected claim 1 because the term "optionally" in the phrase "optionally diluting the suspension" in original claim 1 is allegedly vague and renders the claim indefinite. Applicants respectfully disagree. However, in order to further prosecution and not in acquiescence to the Examiner's objection, Applicants have amended claim 1 to remove the term "optionally". Accordingly, Applicants submit that this rejection has been rendered moot. The same *mutatis mutandis* applies to currently amended dependent claims 2-32.

***B. Rejection of Claim 4 and Claims 5-33***

At page 4 of the Office Action, section 5, 3rd paragraph, the Examiner has rejected claim 4 as well as claims 5-33 because of the presence of multiple dependencies in the claims, which render the claims allegedly indefinite. Applicants have removed the multiple dependencies from the claims. Thus, Applicants respectfully submit that this rejection has been rendered moot.

***C. Rejection of Claim 3***

At page 4 of the Office Action, section 5, last paragraph, the Examiner has rejected claim 3 because the claim *inter alia* recites that multipotent cells are embryonic stem cells, which according to the Examiner is allegedly unclear. Applicants respectfully disagree. However, in order to further prosecution and not in acquiescence to the Examiner's rejection, Applicants have cancelled reference to multipotent cells in claim 3 without prejudice or disclaimer thereof. Thus, Applicants respectfully submit that this rejection has been rendered moot.

***D. Rejection of Claim 4***

At page 5 of the Office Action, section 5, 1st paragraph, the Examiner has rejected claim 4 because of the phrase "derived from", which according to the Examiner allegedly renders the claim indefinite. Applicants respectfully disagree. However, in order to further prosecution and not in acquiescence to the Examiner' rejection, Applicants have amended claim 4 to recite "obtained from" instead of "derived from." Thus, Applicants submit that the rejection has been overcome. Reconsideration and withdrawal are respectfully requested.

***E. Rejection of Claims 5, 6, 19, 21 and 29***

At page 5 of the Office Action, section 5, second paragraph, the Examiner has rejected claims 5, 6, 19, 21 and 29 as being allegedly vague because of the term "and/or" in the claims. Applicants respectfully disagree. However, in order to further prosecution and not in acquiescence to the Examiner's rejection, Applicants have changed the term "and/or" to "... or ... or both," as suggested by the Examiner. Thus, Applicants respectfully submit that this rejection has been rendered moot.

***F. Rejection of Claim 5***

At page 5 of the Office Action, section 5, 3rd paragraph, the Examiner has rejected claim 5 because of the presence of the terms "IMDM" and "FCS", which are considered by the Examiner as allegedly vague and rendering the claim indefinite. Applicants respectfully disagree. However, in order to further prosecution and not in acquiescence to the Examiner' rejection, Applicants have amended claim 5 to spell out the terms "IMDM" and "FCS," as suggested by the Examiner. Thus, Applicants respectfully submit that this rejection has been rendered moot.



***G. Rejection of Claim 12***

At page 5 of the Office Action, section 5, 4th paragraph, the Examiner has rejected claim 12, since according to the Examiner the phrase "wherein the final concentration of EBs in the suspension culture is about 500/ml" renders the claim indefinite, because it is allegedly unclear what unit of "500" is. Applicants respectfully disagree. However, in order to further prosecution, and not in acquiescence to the Examiner's objection, Applicants have amended claim 12 to recite "500 EBs/ml". Thus, Applicants respectfully submit that this rejection has been rendered moot.

***H. Rejection of Claim 25***

At page 5 of the Office Action, section 5, penultimate paragraph, the Examiner has rejected claim 25 because of the presence of the phrase "substantially the same," which allegedly renders the claim indefinite. Applicants respectfully disagree. However, in order to further prosecution and not in acquiescence to the Examiner's objection, Applicants have amended claim 25 to remove the term "substantially". Accordingly, Applicants submit that this rejection has been rendered moot.

***I. Rejection of Claim 26***

At page 5 of the Office Action, section 5, last paragraph, the Examiner has rejected claim 26, since the phrase "selected from different color versions of enhanced green fluorescent protein (EGFP)" according to the Examiner is allegedly vague and unclear. Applicants respectfully disagree. However, in order to further prosecution and not in acquiescence to the Examiner's objection, Applicants have amended claim 26 to remove the term "selected from different color versions of." Accordingly, Applicants submit that this rejection has been rendered moot.

***J. Rejection of Claim 30***

At page 6 of the Office Action, section 5, 1st paragraph, the Examiner has rejected claim 30 for allegedly being indefinite because the terms "alphaMHC" and "MLC2v" are abbreviations that can stand for various meanings. Applicants respectfully disagree. However, in order to further prosecution and not in acquiescence to the Examiner' rejection, Applicants have amended claim 30 by spelling out the terms "alphaMHC" and "MLC2v," as suggested by the Examiner. Thus, Applicants respectfully submit that this rejection has been rendered moot.

***K. Rejection of Claim 32***

At page 6 of the Office Action, section 5, 2nd paragraph, the Examiner has rejected claim 32 because the phrase "derived from" allegedly renders the claim indefinite. Applicants respectfully disagree. However, in order to further prosecution and not in acquiescence to the Examiner' rejection, Applicants have amended claim 32 to recite "obtained from" instead of "derived from." Thus, Applicants submit that the rejection has been overcome. Reconsideration and withdrawal are respectfully requested.

***V. Rejection Under 35 U.S.C. § 112, First Paragraph***

At pages 6-10 of the Office Action, the Examiner has rejected claims 1-33 under 35 U.S.C. § 112, first paragraph, because the specification, while being enabling for producing embryoid bodies (EBs) from pluripotent embryonic stem (ES) cells or embryonic germ (EG) cells, does allegedly not reasonably provide enablement for a method for producing embryoid bodies (EBs) from multipotent cells, including

hematopoietic stem cells, neural stem cells, pancreatic stem cells, follicular stem cells, and any other adult stem cells or progenitor cells, and the production of a differentiated cell which is a cardiomyocyte from said EBs.

Applicants respectfully disagree. However, in order to further prosecution, and not in acquiescence to the Examiner's objection, Applicants have amended claim 1 to recite "[a] method for producing embryoid bodies (EBs) from pluripotent cells."

In addition, Applicants respectfully submit that the Examiner's assertion at page 9 of the Office Action, last paragraph, "that only pluripotent embryonic stem cells or embryonic germ cells can produce EBs" is not correct. Rather, and this is correctly noted by the Examiner, it is the pluripotent state of a cell, which renders it capable of generating embryoid bodies (EBs), which is particularly illustrated by the recent publications reporting on the further developments of inducing pluripotency of somatic cells (iPS cells) which are capable of producing EBs. Indeed, further search of the state of the art of generating EBs reveals that also non-embryonic cells are capable of generating embryoid bodies (EBs). For example, reference is made to international application WO03/018780, published before the filing date of the present application, which describes a method for effecting the de-differentiation of a somatic cell into a pluripotent state, wherein the resultant de-differentiated cells are capable of forming EBs. *See* the abstract of WO03/018780, copy of the international application enclosed herewith (Exhibit A). The finding of de-differentiation and reprogramming of adult somatic cells into a pluripotent state has been confirmed recently. *See, e.g.*, the most recent publication by Kim *et al.*, *Nature*, published online on June 29, 2008, copy enclosed herewith (Exhibit B).

Furthermore, the induction of stem cell-like plasticity in mononuclear cells derived from unmobilized adult human peripheral blood cells is described in Abuljadayel, *Curr. Med. Res. Opin.* 19:355-375 (2003), copy of which is enclosed herewith (Exhibit C), wherein the resultant cells form embryoid body (EB)-like structures. *See* the abstract of Abuljadayel.

In addition, reference is made to international application WO03/052080, copy of which is enclosed herewith (Exhibit D), which describes a method for deriving precursors to human pluripotent non-embryonic stem (P-PNES) and human pluripotent non-embryonic stem (PNES) cell lines, which cells are capable of generating EBs.

In view of the forgoing discussion, Applicant submits that a person having ordinary skill in the art, in view of the teachings of the specification and the knowledge in the art, would be able to make and practice the full scope of Applicant's claimed invention. Accordingly, Applicant respectfully requests that the rejection of the claims under 35 U.S.C. § 112, first paragraph, be reconsidered and withdrawn.

***VI. The Rejections Under 35 U.S.C. § 102 are Traversed***

***A. Rejection of Claims 1-3 and 31***

At pages 10 and 11 of the Office Action, the Examiner has rejected claims 1-3 and 31 under 35 U.S.C. § 102(e) as allegedly being anticipated by Thomson *et al.*, 2003 (U.S. Patent No. 6,602,711 B1; hereinafter "Thomson"). Applicants respectfully disagree. However, solely in an effort to advance prosecution, and not in acquiescence to any reasoning underlying the Examiner's rejection, Applicants have amended claim 1. In particular, it has been clarified that the suspension culture of pluripotent cells is a

single cell suspension. As correctly noted by the Examiner, Thomson teaches a method for producing embryoid bodies from embryonic stem cells in the form of "**clumps**". Thus, contrary to the claimed invention, Thomson does not teach a method of producing embryoid bodies from a single cell suspension, *i.e.*, from single individual cells, but from cell aggregates, *i.e.*, clumps. In particular, Thomson describes the generation of ES cell aggregates by partially dissociating overgrown or piled cultures of ES cells into clumps, which are then cultured in suspension to induce further differentiation. Thomson does not teach treatment of the cells in order to obtain a single cell suspension. Rather, Thomson teaches that "[s]ome dissociation of the colonies occurs, but this is not sufficient to individualize the cells." *See* Thomson at column 4, lines 9-10.

Furthermore, to avoid adhesion of the aggregates to the surface of the cell culture dish, Thomson suggests incubation on a rocking table or a non-adherent plastic dish. Clearly, the method described in Thomson uses the rocking table not for generation of aggregates themselves, but rather only to prevent the already existing aggregates from sticking to the surface during culture. In contrast, as illustrated in Example 1, the pluripotent stem cells subjected to the method of the present invention are present as a single cell suspension which is agitated until formation of the cell aggregates and EBs. *See* specification at page 42, lines 15-26.

Finally, Thomson does not teach a cell concentration of  $0.5 \times 10^6$  to  $5 \times 10^5$  cells/ml or  $0.1 \times 10^6$  to  $1 \times 10^6$  cells/ml as recited in present claims 1 and 45, respectively.

Under 35 U.S.C. § 102, a claim can only be anticipated if every element in the claim is expressly or inherently disclosed in a single prior art reference. *See Kalman v.*

*Kimberly Clark Corp.*, 713 F.2d 760, 771 (Fed. Cir. 1983), *cert. denied*, 465 U.S. 1026 (1984). As discussed above, Thomson does not expressly or inherently disclose every element of the presently claimed invention. Hence, under *Kalman*, this reference cannot support a rejection under 35 U.S.C. § 102(e). In view of the foregoing remarks, Applicants respectfully assert that Thomson does not anticipate the currently presented claims. Reconsideration and withdrawal of the rejection under 35 U.S.C. § 102(e) over Thomson therefore are respectfully requested.

***B. Rejection of Claims 1, 3 and 31***

At pages 11 and 12 of the Office Action, the Examiner has rejected claims 1, 3 and 31 under 35 U.S.C. § 102(a) as allegedly being anticipated by Dang *et al.*, June 20, 2003 (US 2003/0119107 A1; "hereinafter Dang"). Applicants respectfully traverse this rejection with regard to the currently amended claims and the newly added claims.

The Examiner asserts that "Dang teaches a method for efficient formation of EBs and the culture of EBs in suspension at higher cell densities by controlling cell aggregation via stirring *or agitation* of the liquid suspension." (emphasis added) See Office Action at page 12. Applicants respectfully disagree with this statement

Indeed, Dang does not come even remotely close to the present invention. In particular, despite the Examiner's assertion to the contrary, Dang does not teach agitation as an alternative means for controlling aggregation of EBs. A closer look at paragraph [0053] of Dang, the only paragraph mentioning agitation, reveals that agitation can be used as an alternative to stirring to keep cells and/or spheroids in liquid suspension.

There is no mentioning whatsoever of generating aggregates or EBs by agitation of a liquid single cell suspension culture.

Thus, contrary to the impression the Examiner may have had on first reading of Dang, the option of agitating the culture system is only related to keeping individual cells and/or spheroids in liquid suspension but NOT for generating and controlling the aggregation of EBs. As the Federal Circuit has held, a claim can only be anticipated by a publication if the publication describes the claimed invention with sufficient enabling detail to place the public in possession of the invention. *See In re Donohue*, 766 F.2d 531, 533 (Fed. Cir. 1985); *see also PPG Industries, Inc. v. Guardian Industries Corp.*, 75 F.3d 1558, 1566 (Fed. Cir. 1996) ("To anticipate a claim, a reference must disclose every element of the challenged claim and enable one skilled in the art to make the anticipating subject matter."). Since Dang does not disclose or teach how one of ordinary skill would use agitation to promote aggregation, this reference does not enable one of ordinary skill to make the subject matter of the presently claimed invention. Accordingly, for at least these reasons, and under *Donohue* and *PPG Industries*, Dang cannot and does not anticipate the present claims. Reconsideration and withdrawal of the rejection under 35 U.S.C. § 102(a) over Dang therefore are respectfully requested.

**VII. Rejections Under 35 U.S.C. § 103(a) are Traversed**

**A. Rejection of Claims 1 and 4-6**

At pages 13-15 of the Office Action, the Examiner has rejected claims 1 and 4-6 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Thomson in view of Dang *et al.*, *Biotechnol. Bioeng.* 78:442-453 (2002) ("hereinafter Dang *et al.*"). Applicants

respectfully traverse this rejection with regard to the currently amended claims and the newly added claims.

The Examiner acknowledges that Thomson does not teach using murine ES cells, IMDM culture medium, 20 % FCS and 95% humidity. However, the Examiner asserts that because Dang teaches culturing CCE murine embryonic stem cell in IMDM medium at 37°C in humidified air with 5% CO<sub>2</sub> and the use of liquid suspension cultures of murine ES cells from EBs:

It would have been prima facie obvious to one of ordinary skill in the art at the time of the invention to culture murine ES cells in IMDM medium at 37°C in humidified air with 5% CO<sub>2</sub> because Dang teaches culturing murine ES cells in said condition. In also would have been prima facie obvious for one of ordinary skill in the art to culture ES cells in medium having 20% FCS and 95% humidity because Thomson teaches culturing ES cells in medium having 20% FBS and both Thomson and Dang teach culturing ES cells in humidified condition.

Office Action at page 14. Applicants respectfully disagree.

The factors to be considered under 35 U.S.C. § 103(a), are the scope and content of the prior art; the differences between the prior art and the claims at issue; and the level of ordinary skill in the pertinent art. *See Graham v. John Deere*, 86 S.Ct. 684 (1966) and MPEP §2141. This analysis has been the standard for 40 years, and remains the law today. *See KSR International Co v. Teleflex Inc.*, 127 S.Ct. 1727 (2007). The Office has recently published Examination Guidelines to aid Examiners in formulating obviousness rejections. *See Examination Guidelines for Determining Obviousness under 35 U.S.C. 103 in view of the Supreme Court decision in KSR International v. Teleflex Inc.* Fed. Reg. Vol. 72, pp. 57526 to 57535 (October 10, 2007), hereinafter "the Examination Guidelines." Seven rationales are suggested by which obviousness may be found, *e.g.*,



by combining elements in the art or substituting one known element for another. As a common thread through all the rationales, the Examiner must establish on the record that a person of ordinary skill in the art would have recognized that the results of the combination or substitution were *predictable*. *Id.*, *e.g.*, at 57529.

The Examiner has not met the burden of establishing a *prima facie* case of obviousness based on the Examination Guidelines. Specifically, the Examiner has not established that the ordinary artisan reading Thomson and Dang *et al.* in combination would arrive at the presently claimed method that uses agitation of a liquid single cell suspension culture of pluripotent cells to generate cell aggregates and EBs.

Thomson has been discussed in detail above. Dang *et al.* discloses maintenance and differentiation of CCE murine embryonic stem cells in liquid suspension cultures. However, Dang *et al.* does not teach or suggest that the liquid suspension culture should be agitated to generate cell aggregates and EBs, respectively. Furthermore, Dang *et al.* does not teach nor suggest that the pluripotent cells are present at a concentration of  $0.5 \times 10^6$  to  $5 \times 10^5$  cells/ml or  $0.1 \times 10^6$  to  $1 \times 10^6$  cells/ml, respectively. Rather, Dang *et al.* teaches liquid suspension cultures with a desired ES cell concentration of  $\leq 10^3$  cells/ml or between  $10^2$  and  $10^5$  cells/ml. See Dang *et al.* at page 444, right column, lines 1-5 and at page 446, left column, Fig.2 and the description under the section "EBs Aggregate, Reducing EB Formation Efficiency" up to the right column, line 15. Thus, the combination of the references does not provide the combination of the elements of (i) agitating a liquid single cell suspension culture of pluripotent cells with a (ii) concentration of  $1 \times 10^6$  to  $5 \times 10^5$  cells/ml or  $0.1 \times 10^6$  to  $0.5 \times 10^6$  cells/ml (iii) until the formation of EBs.

In contrast to the cited references, the present invention provides for the production of embryoid bodies in large amounts and high density by agitating a certain amount and concentration of pluripotent cells. *See* specification at page 9, lines 6-14. Applicants respectfully assert that the ordinary artisan reading the combination of references could not have predictably arrived at the presently claimed invention of producing embryoid bodies (EBs) from pluripotent cells by agitation of a liquid single cell suspension culture of pluripotent cells with a concentration of  $0.5 \times 10^6$  to  $5 \times 10^5$  cells/ml or  $0.1 \times 10^6$  to  $1 \times 10^6$  cells/ml to generate cell aggregates and EBs. Neither reference cited by the Examiner teaches using agitation to generate cell aggregates and EBs. Therefore, Applicants submit that the Examiner has failed to establish a *prima facie* case of obviousness as set out in the Examination Guidelines and respectfully request that this rejection be withdrawn.

***B. Rejection of Claims 1, 7-18, 32 and 33***

At pages 15-17 of the Office Action, the Examiner has rejected claims 1, 7-18, 32 and 33 under 35 U.S.C. § 103(a) as allegedly being obvious over Thomson in view of Kehat *et al.*, 2001 (The Journal of Clinical Investigation, Vol. 108, No. 3, p. 407-414; “hereinafter Kehat”). As indicated above, Applicants have cancelled claims 14-16 and 18 without prejudice or disclaimer thereof, thus rendering moot the rejection as it applied to these claims. Applicants respectfully traverse this rejection with regard to the remaining claims and the newly added claims.

The Examiner acknowledges that Thomson does not teach “a cell concentration of  $1 \times 10^6$  to  $5 \times 10^5$  cells/ml or  $0.1 \times 10^6$  to  $0.5 \times 10^6$  cells/ml, 1:10 dilution, final

concentration of EBs in the suspension culture and culturing the suspension culture for about 6hr or 16-20hr.” *See* Office Action at page 16. However, the Examiner alleges that it would have been *prima facie* obvious for one of ordinary skill in the art at the time of the invention to culture ES cells at a concentration of  $1 \times 10^6$  to  $5 \times 10^5$  cells/ml or  $0.1 \times 10^6$  to  $0.5 \times 10^6$  cells/ml, 1:10 dilution, with a certain final concentration of EBs in the suspension culture, and culturing the suspension culture for about 6hr or 16-20 hr, because Thomson teaches culturing the ES cells for at least 48 hours and up to 14 days and Kehat teaches culturing ES cells at about  $5 \times 10^6$  cells in a 58 mm dish. Applicants respectfully disagree with this conclusion.

Thomson has been discussed in detail above. Kehat, like Thomson, teaches using **clumps** of ES to generate EBs, not a **single cell** suspension, as presently claimed. *See* Kehat at page 408, the paragraph bridging left and right column. Furthermore, the culture taught in Kehat is a static culture. *See* Fig. 1 of Kehat at page 408 and the figure legend. Nowhere in Kehat is it taught or suggested that the cell suspension culture should be agitated in any way to produce aggregates and EBs, respectively.

Thus, the ordinary artisan reading the combination of references cannot predictably arrive at the presently claimed invention of producing embryoid bodies (EBs) from pluripotent cells by agitation of a liquid single cell suspension culture with a concentration of  $0.5 \times 10^6$  to  $5 \times 10^5$  cells/ml or  $0.1 \times 10^6$  to  $1 \times 10^6$  cells/ml until generation of cell aggregates and EBs. Specifically, the references do not disclose the elements of *agitating a liquid single cell suspension culture* of pluripotent cells to generate cell aggregates and EBs. Therefore, Applicants submit that the Examiner has

failed to establish a *prima facie* case of obviousness and respectfully request that this rejection be withdrawn.

**C. Rejection of Claims 1 and 19-30**

At pages 17-20 of the Office Action, the Examiner has rejected claims 1 and 19-30 under 35 U.S.C. § 103(a) as allegedly being obvious over Thomson in view of Dang. Applicants respectfully traverse this rejection with regard to the currently amended claims and the newly added claims.

The Examiner asserts that

It would have been *prima facie* obvious for one of ordinary skill in the art at the time of the invention to prepare ES cells expressing a selectable marker, such as puromycin resistant gene, and/or a reporter gene, such as EGFP, under the control of a cell type-specific regulatory sequence because Dang teaches using a ES cell expressing GFP under the control of constitutively active promoter and GFP is a type of selectable marker, and one of ordinary skill would use another selectable marker or a cell specific promoter in order to optimize the expression of the marker at target cells and to optimize visualization or detection of the ES cells.

Office Action at pages 19-20. We respectfully disagree with this statement.

Thomson and Dang have been discussed in detail above. Neither Thomson nor Dang discloses agitating a liquid single cell suspension culture *to generate* aggregates and EBs. As discussed above, the method described in Thomson uses a rocking table not for generation of aggregates themselves, but rather only to prevent the already existing aggregates from sticking to the surface during culture. Likewise, Dang only suggests agitation as a method to keep individual cells and/or spheroids in liquid suspension.

Thus, the ordinary artisan reading the combination of references cannot predictably arrive at the presently claimed invention of producing embryoid bodies (EBs) from pluripotent cells by agitating a liquid single cell suspension culture of pluripotent cells with a concentration of  $0.5 \times 10^6$  to  $5 \times 10^5$  cells/ml or  $0.1 \times 10^6$  to  $1 \times 10^6$  cells/ml to generate cell aggregates and EBs. Specifically, these references do not disclose the element of using agitation to generate aggregates and EB's. Therefore, Applicants submit that the Examiner has failed to establish a *prima facie* case of obviousness and respectfully request that this rejection be withdrawn.

#### ***VIII. Conclusion***

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

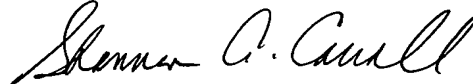
Amdt. dated March 11, 2009 - 28 -  
Reply to Office Action of December 11, 2008

KOLOSSOV *et al.*  
Appl. No. 10/594,188

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

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WO 03/018780 A1

(54) Title: DE-DIFFERENTIATION AND RE-DIFFERENTIATION OF SOMATIC CELLS AND PRODUCTION OF CELLS FOR CELL THERAPIES

(57) Abstract: The invention provides a method for effecting the de-differentiation of a somatic cell by culturing the cell in the absence of growth factors, cytokines, or other differentiation-inducing agents, and introducing components of cytoplasm of pluripotent cells into the somatic cell and allowing the cell to de-differentiate. The method can be used with somatic cell and allowing the cell to de-differentiate. The method can be used with somatic cells of any type, from any species of animal. The pluripotent cells may be oocytes, blastomeres, inner cell mass cells, embryonic stem cells, embryonic germ cells, embryos consisting of one or more cells, embryoid body (embryoid) cells, morula-derived cells, teratoma (teratocarcinoma) cells, as well as multipotent partially differentiated embryonic stem cells taken from later in the embryonic development process. After being de-differentiated, the cell can be induced to re-differentiate into a different somatic cell type. A method for de-differentiating a somatic cell and inducing it to re-differentiate into a cell of neural lineage is disclosed.

## DE-DIFFERENTIATION AND RE-DIFFERENTIATION OF SOMATIC CELLS AND PRODUCTION OF CELLS FOR CELL THERAPIES

### Related Applications

**[0001]** This application claims benefit of priority to U.S. Serial No. 60/314,657 filed on August 27, 2001 which is incorporated by reference in its entirety herein.

### Field of the Invention

**[0002]** The present invention provides novel methods for de-differentiating adult somatic cells into multi-potential stem-like cells without generating embryos or fetuses. Cells developed using the present invention can then be differentiated into neuronal, hematopoietic, muscle, epithelial, and other cell types. These specialized cells have medical applications for treatment of degenerative diseases by "cell therapy".

### Background

**[0003]** Today, the vast majority of degenerative diseases are treated by drugs or symptomatic therapies (e.g., alleviation of pain) due to lack of available patient-compatible cells or tissues that could replace damaged tissue or repair the lesions induced by a given disorder. Current cell-based therapeutic approaches being developed involve either allogeneic cells derived from human embryonic stem cells or xenogeneic cells derived from pigs. Examples of these approaches for Parkinson's disease are differentiated human neurons (Geron) and fetal pig neural cells (Diacrin). Although these strategies hold scientific promise, they suffer from major limitations. First, there is considerable controversy over the use of human embryos for stem cell research and development. Second, the use of pig cells suffers from potentially unknown issues involving the transmission of porcine-borne pathogens to humans. Third, both of these strategies require the use of immuno-suppression, which increases the risk of infections.

**[0004]** There is at present a great need for an efficient method to derive multi-potential stem-like cells from a patient's own somatic cells. For example,



15.7 million people (5.9% of the population) in the United States have diabetes. Each day approximately 2,200 new cases of diabetes are reported, and nearly 800,000 people will be diagnosed this year. Diabetes is the seventh leading cause of death in the United States and is a chronic disease that has no cure. Debilitating medical conditions caused by diabetes include kidney failure, blindness, heart attack, and stroke. It costs an estimated \$140 billion per year to treat diabetes-related illnesses in the United States. It is more difficult to predict the indirect costs of the disease, which are those associated with worker productivity and societal contributions. Autologous cell therapy, which would replace lost pancreatic cells in a single medical procedure, could eliminate most of these costs. The present invention offers a means to cure, not just treat, the disease. Furthermore, the ability to de-differentiate somatic cells to a multi-potential state, provides the opportunity to treat many of the secondary illnesses associated with diabetes as well. The advantage of the present invention over other allogeneic cell therapy-based approaches is a further reduction in complications and associated costs of histo-incompatibility. Of course, the most immediate and vital benefit of the cell therapies made possible by present invention, although not quantifiable, is the unprecedented improvement in quality of life for patients suffering from incurable degenerative diseases.

#### Description of the Drawings

**[0005]** Figure 1: Proliferating bovine adult skin fibroblasts growing on 100 mm tissue culture dishes at about 90% confluence.

**[0006]** Figure 2: Colonies formed by bovine adult fibroblasts four days after the cells were electroporated with high speed xenopus oocyte extract; the cell colonies are morphologically similar to embryonic stem cell colonies.

**[0007]** Figure 3A: Cells derived from bovine adult fibroblasts electroporated with *Xenopus* oocyte extract - the cells are beginning to display a neuronal phenotype with a "phase bright" appearance of the cell body.

**[0008]** Figure 3B: Bovine fibroblast-derived cells that are beginning to display a neuronal phenotype.

**[0009]** Figure 4: Bovine fibroblast-derived cells with a neuronal phenotype and axonal-like processes. The cells were obtained by culturing the cells shown in Figs. 3A/B for 3 days in DMEM/F12 ITS with 10  $\mu$ g/ml Nerve Growth Factor.

**[0010]** Figure 5: Bovine fibroblast-derived cells with a neuronal phenotype and axonal-like processes that appear to be in contact with one another. The cells were obtained by culturing the cells shown in Figs. 3A/B for 3 days in DMEM/F12 ITS with 10  $\mu$ g/ml Nerve Growth Factor. (Figure 5).

**[0011]** Figure 6: Bovine fetal pancreas primary cell culture 3 days after isolation. Cells either plated down (A) or remained in suspension in aggregates (B). Pancreatic cells four weeks after initiation of culture (C). Bovine fibroblast primary cell cultures (controls, D) were dissociated by trypsinization and electroporated with CytoTracker Blue (Molecular Probes, Eugene, OR) prelabeled bovine oocyte lysate. After the electroporation, cells were plated on gelatin coated cell culture dishes and examined for the presence of CytoTracker Blue 24 hours later (E-phase, F-fluorescence using UV excitation). After 1 week in culture, the cells started forming colonies resembling stem cell aggregates (G), which increased in size during the following 2 weeks (H, I). All images were taken at 100X, recorded with DAGE-MTI camera and printed on a UVP printer. Images were scanned into Adobe Photoshop and pseudo-colored.

**[0012]** The remaining cells were plated in 3 replicate 60 mm dishes of cells. After 3 days, the medium was changed to 1) DMEM/F12 ITS; 2; and 3) Neurobasal Medium A (NBA, Clonetics) with 10  $\mu$ g/ml NGF. The cells treated with DMEM/F12 ITS alone displayed a phenotype similar to that observed before.

### Description of the Invention

**[0013]** The present invention exploits the fact that all of the somatic cells of an individual contain the genetic information required to become any type of cell, and when placed into a conducive environment, a terminally differentiated cell's fate can be redirected to pluripotentiality. This fact has been exemplified by the success of somatic cell nuclear transfer experiments in non-human mammals. As normal development proceeds, the gene expression profile of a cell becomes restricted and regions of the genome are stably inactivated such that, under normal conditions, the cell cannot rejuvenate. It is well-established that cell type-specific gene expression can be altered by environmental insults (as in wound healing, bone regeneration, and cancer). The present invention provides cells with intracellular and environmental clues that will induce changes in nuclear function and consequently, change the cell's identity. Using the present invention, cytoplasm from known pluripotent cell types, such as human teratocarcinoma cells, spermatogonia, mature frog, and mammalian oocyte cytoplasm extract is incorporated into somatic cells by electroporation or by BioPorter® (Gene Therapy Systems, San Diego, CA). After incorporation, cells are cultured using conditions that support maintenance of de-differentiated cells (i.e. stem cell culture conditions). The dedifferentiated cells can then be expanded and induced to re-differentiate into different type of somatic cells that are needed for cell therapy; for example, into glucose responsive, insulin-producing pancreatic beta cells.

**[0014]** The present invention permits the memory of an adult differentiated somatic cell to be replaced with its long forgotten embryonic memory by manipulating the intra- and extra-cellular environment. By providing an adult somatic cell with factors present in mature oocyte cytoplasm and/or factors present in other known pluripotent cell types (e.g., spermatogonia, teratocarcinoma cells), the invention restores the cells' epigenetic memory to a state similar to that of pluripotent stem cells (without creating an embryo). The invention provides a means for (1) determining the minimal effective quantity of oocyte cytoplasmic lysate/extract required for reprogramming, and (2)

preparing high-speed extracts from lysates to eliminate the mitochondrial and nuclear contribution from the "reprogramming matrix" and make it semi-defined. The high-speed extract can be fractionated and individual fractions tested for reprogramming ability, leading to development of a product for reprogramming somatic cells.

**[0015]** In practicing the present invention, no embryos or fetuses of any species are ever created or used and no mixing of human and non-human mitochondrial or genomic DNA ever occurs. All the methods of the invention can be performed in vitro and sources of reprogramming cytoplasm are available from local slaughterhouses (bovine oocytes and spermatocytes), *Xenopus* oocytes (in house, IACUC approved), or from commercial sources (teratocarcinoma cells from ATCC).

**[0016]** The object of the present invention is to develop technology to change the nuclear function of one type of highly specialized somatic cells, e.g. skin fibroblasts, into that of another type, e.g., fully functional pancreatic islets, via a "novel" pluripotent cell intermediate. The invention does not utilize embryonic or fetal tissues to accomplish the change in function and can be designed for individual patients using their own cells.

**[0017]** The invention exploits the fact that all of the cells of an individual contain the genetic information required to be expressed by any cell type when placed into a conducive environment (as shown by somatic cell nuclear transfer experiments). Most of this information becomes repressed as differentiation proceeds and remains stably inactivated in all differentiated cell types. It is well established that expression of cell type-specific genes is determined by environmental signals and can be altered by environmental insults (as in wound healing, bone regeneration, and cancer). The present invention provides cells with intracellular and environmental clues that will induce change of nuclear function and consequently change cells' identity. In one embodiment of the invention, cytoplasmic extract from known pluripotent cell types, such as human teratocarcinoma cells, spermatogonia, and mature frog and mammalian oocytes, is delivered into somatic cells by electroporation or by BioPorter® (Gene Therapy Systems, San Diego, CA). After delivery, the cells are exposed

to an environment that supports de-differentiated cell types; e.g., stem cell culture conditions. Upon expansion to numbers sufficient for several differentiation pathways, the cells are directed to re-differentiate; for example, into pancreatic islet cells.

**[0018]** As shown by the success of somatic cell nuclear transfer, the ability to erase the memory of an adult differentiated somatic cell and replace it with its long forgotten embryonic memory is limited only by the ability to manipulate the intra- and extra-cellular environment. By providing the nucleus of an adult somatic cell with factors present in mature oocyte cytoplasm (without creating an embryo) and/or factors present in other known pluripotent cell types (spermatogonia, teratocarcinoma cells), the present invention alters nuclear memory and induces nuclear changes that are commonly observed in pluripotent stem cells. Benefits and advantages of the invention include the following:

- (i) No need for human embryos or fetal tissue. With the present invention, embryos do not have to be used, created, or destroyed to generate pluripotent cells, thus eliminating ethical concerns.
- (ii) No need for patient immuno-suppression. In most cases, extended graft survival can only be expected when combined with pharmaceutical immuno-suppression. A preferred method of long-term and lasting treatment using cell-based therapy is to use cells originally derived from the patient.
- (iii) No health risks due to possible transmission of animal viruses. Since no component of the animal genome is ever used in the invention, potential threats due to animal genomic DNA sequences are not a concern.
- (iv) No mitochondrial incompatibility. Mitochondrial DNA is removed from the reprogramming matrix by ultracentrifugation.
- (v) No need for pharmacological therapy. Cell transplantation can be used alone and does not have to be supported by any pharmacological agents.
- (vi) Few or no side effects. Autologous cell transplantation is unlikely

to induce adverse side effects.

(vii) No tolerance / resistance induction by therapy. Autologous cell transplants are not expected to induce resistance and if required, repeated cell transplantation is feasible.

(viii) Short cell generation time. This invention contrasts with embryonic methods, which have yielded only small numbers of starting stem cells (between 10-15 cells from a blastocyst). Since large numbers of cells can be harvested from individual patients (a single, common source of stem cells is not required any longer) as starting material, the degree of in vitro proliferation is only what is needed to de-differentiate them and generate enough cells for the clinical application.

(ix) Cure, not only treatment. The present invention will significantly reduce the cost of cell therapy by eliminating the need for immunosuppression of the patient to reduce acute and hyperacute rejection. The need for repeated transplantation procedures will also be alleviated, reducing the indirect cost of disease treatment.

(x) Model. Presumptive human pancreatic beta cells can be tested by transplantation into SCID mice as described (Lanza et al., 1997) and do not require a non-human primate model.

#### Abbreviations used in Application

3-D – three dimensional

5'UTR – 5'untranslated region

ACT – Advanced Cell Technology

Alpha 1AT – alpha 1 anti-trypsin

ANOVA – analysis of variance

ATCC – American Type Culture Collection

bFGF – basic fibroblast growth factor

bHLH – basic-helix-loop-helix

CAMs – cell adhesion molecules

CDk2 cell cycle kinase

DMEM – Dulbecco modified minimum essential medium  
DMSO – dimethylsulfoxide  
DTZ – dithizone  
EGM – endothelial growth medium  
E1A – adenoviral protein  
EC – extra cellular  
FACS – fluorescence assisted flow cytometry sorting  
FCS – fetal calf serum  
FFA – free fatty acids  
G0/G1 – gap phases of the cell cycle  
GCT44 – human yolk sack teratoma cell factor  
GFP – green fluorescent protein  
H1 – histone H1  
HDL – high-density lipoproteins  
HDM – hormone-defined medium  
HGF – hepatocytes growth factor  
HGM – hepatocytes growth medium  
HPLC – High performance liquid chromatography  
IACUC – Institutional Animal Care and Use Committee  
IAPP – anti-islet amyloid peptide  
ICC – immunocytochemistry  
IVF – in vitro fertilization  
Kb – kilobase  
LDL – low-density lipoproteins  
LIF – leukemia inhibiting factor  
LN<sub>2</sub> – liquid nitrogen  
NGF – nerve growth factor  
NuMA – nuclear matrix associated protein  
Oct4GFP – a transgene: Oct4 promoter (transcription factor) driving GFP  
(Green Fluorescent Protein) expression  
PEG – polyethylene glycol  
PERVS – Porcine endogenous retroviruses

PL - phospholipids

RT-PCR – reverse transcription-polymerase chain reaction

SCID – severe combined immune deficiency

TRITC – isothiocyanate

**[0019]** This invention essentially provides a method for de-differentiation of one type of somatic cells into pluripotent stem-like cells using a semi-defined cell-free system in vitro. The invention provides a cell-free reprogramming matrix that will reliably direct de-differentiation of adult differentiated human cells into a stem-like cell type. Stem-like cells are then induced to differentiate into desired somatic cell type. This process provides autologous (isogeneic) cell types for cell transplantation in the same individual that donated the initial somatic cell sample. The present invention circumvents problems of histoincompatibility that exists with competing cell therapy strategies, and shortens significantly the time required for the “new” cells to be available for therapy and does not use embryo or fetus intermediaries as vehicles for reprogramming. The invention also includes methods for characterization and maintenance of the newly de-differentiated cells, stable cell morphology and analysis of cell-specific gene and protein expression; and induced re-differentiation into cells of another type.

**[0020]** The present invention provides for efficient reprogramming and de-differentiation of somatic cells; maintenance of de-differentiated state in vitro; determining the ability of cells to differentiate upon induction, and the assessment of newly induced differentiated cell types to exhibit proper function upon cell transplantation. Aspects of the invention include characterizing both de-differentiated and newly induced cell types for their gene expression, protein expression, secretory function, presence of cell surface antigens, ability to proliferate, and karyotype stability. Specific aspects of the invention are described in detail below.

Preparing and characterizing high-speed extracts (reprogramming matrix)

**[0021]** Components of reprogramming machinery are clearly present in



mature, metaphase II arrested mammalian oocytes, as shown by the successes of nuclear transplantation experiments. Various types of adult somatic nuclei from several species have been reprogrammed using an oocyte cytoplasm where the nucleus acquired totipotency, and reconstructed embryos developed into healthy offspring upon transfer into recipient animals (reviewed by Pennisi and Vogel, 2000). An approach to conceptually related to reprogramming after nuclear transfer into oocytes is the study of changes in nuclear function that occur after the fusion of two distinct somatic cell types into a heterokaryon. A gene that is normally active only in a given cell is often inactivated upon fusion of that cell with a different type of cell or with an undifferentiated cell (Kikyo and Wolffe, 2000). Similarly, activation of a new gene can occur by induction of pluripotent cell-specific transcription factors that in turn might activate a diverse group of genes downstream (Hardeman et al., 1986).

**[0022]** *Xenopus* extracts have been used extensively for examination of mammalian somatic cell gene activity during the past 40 years. After incubation of a nucleus in oocyte extracts, a considerable amount of protein is taken up into the nucleus (Merriam, 1969). This is accompanied by nuclear swelling and a decrease in the amount of heterochromatin in the somatic nucleus. Remarkably, over 75% of pre-existing somatic nuclear protein is lost, probably due to the active oocyte nucleoplasmin. In addition to nucleoplasmin, energy-dependent chromatin remodeling machinery is probably required for reprogramming nuclei (Blank et al., 1992). Such energy-dependent process may involve ATPases, DNA polymerases, or dedicated chromatin-remodeling machines, such as SWI2/SNF2 superfamily. Indeed, it has been shown that nucleosomal ATPase ISWI has an important role during this process (Kikyo et al., 2000). The results of experiments of these and other researchers suggest that cells maintain continuous regulation of a plastic differentiated state in which all of the genes are continually regulated by trans-acting factors that either activate or repress their transcription. (Blau and Baltimore, 1991). The process of transcription requires considerable remodeling of chromosomal structure, such as that which occurs in *Xenopus* egg cytoplasm (Kikyo and

Wolffe, 2000). The present invention demonstrates that reprogramming matrix components can be isolated in a semi-pure protein complex form from oocytes and pluripotent cell types and used to revert nuclear function of somatic cells.

Preparation of high-speed metaphase II *Xenopus* oocyte extract.

**[0023]** Mature *Xenopus* oocytes are obtained from superovulated female frogs and low and low speed and high-speed extracts can be prepared as described (Blow and Laskey, 1986). Oocytes are placed in High Salt Barth solution (110 mM NaCl, 2 mM KCl, 1 mM MgSO<sub>4</sub>, 0.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM NaHCO<sub>3</sub>, 15 mM Tris-HCl, pH 7.4) and processed within 2 hours. The eggs are dejellied in 2% cystein (pH 7.8) and washed several times in 20% modified Barth Solution (20% MBS: 18 mM NaCl, 0.2 mM KCl, 0.5 mM NaHCO<sub>3</sub>, 2 mM Hepes-NaOH, pH 7.5; 0.15 mM MgSO<sub>4</sub>, 0.05 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>). The eggs may then be activated for preparation of interphase extract (e.g., by 0.5 µg/ml Ca-ionophore A23187 for 5 min), or used un-activated for the extract preparation. They are washed in ice-cold extraction buffer: 50 mM Hepes-KOH (pH 7.4), 50 mM KCl, 5 mM MgCl<sub>2</sub>, 2 mM β-mercaptoethanol, 3 µg/ml leupeptin and 10 µg/ml cytochalasin B. Washed eggs are pooled into cooled centrifuge tubes, the excess buffer is removed and the eggs are crushed by centrifugation in a swinging bucket rotor (e.g., Sorvall® AH-650) at 9,000 rpm at 4°C for 15 minutes. This produces 4 major fractions: a dense insoluble plug of yolk platelets and pigment, a golden-brown cytoplasmic layer, a lighter translucent cytoplasmic layer, and a yellow plug of lipid. The golden colored cytoplasmic layer is removed with a cooled Pasteur pipette and centrifuged in the same rotor at 9,000 rpm at 4°C for 15 minutes again in order to remove residual debris. The final protein concentration in the extract ranges around 45 mg/ml. High speed extract is prepared from the golden cytoplasmic layer by centrifugation at 100,000g for 60 minutes. A translucent pellet of polyribosomes and glycogen is found at the bottom of the tube. Heavy membranes sediment above. The cytoplasmic layer is removed and used to in the procedures to effect de-differentiation. To preserve cellular proteins and their activity, all the procedures are carried out at 4°C.

**[0024]** Extracts are prepared from bovine oocytes, teratocarcinoma cells and

spermatogonial cells using similar methods. Every batch of extract is screened for the presence of genomic and mitochondrial DNA by Hoechst 33342 and MitoTracker DNA staining.

**[0025]** Protein content of extracts is determined by established protocols (BioRad®, Hercules, CA). The extract is fractionated by HPLC using Superdex® column, which separates proteins based on their size and shape. Each fraction is collected and tested individually for its reprogramming activity.

#### Collecting and analyzing data.

**[0026]** The extracts can be characterized for the presence of molecules that have been shown in intact oocytes to be important during normal fertilization and embryonic development. For example, levels of histone H1 kinase cdc2 (relating to preservation of the metaphase state) and MAP2 kinase and their dynamics and persistence in cell-free extracts prior to hybridization by Western blotting can be determined, as well as quantities and the phosphorylation state of CDK2, cyclin A, Cyclin B, cyclin E, cdc25, p53, nucleoplasmin, histones, RNA and DNA polymerases, Oct4 transcription factor and E1A-like protein, which can be routinely monitored by Western blotting. The molecular profile of each batch of extract can be standardized so that known dilutions of proteins/activity are present in the hybridization matrix. A minimum effective dose is determined as that giving 50% of hybridized cells showing change of nuclear function (down-regulation of donor cell-specific genes) within 48 hours, and by induction of Oct4GFP fluorescence.

#### Delivery of extracts into patient's somatic cells.

**[0027]** In order to introduce large molecules into living cells, the plasma membrane needs to be perturbed. There are several published protocols that can achieve this goal with various degrees of efficiency; for example, electric fusion, electroporation, polyethylene glycol treatment (PEG), and liposomes are some of these protocols. In addition, the following two approaches can be used to effect extract delivery:

1. The BioPorter® protein delivery reagent (Gene Therapy Systems, Inc.) is a unique lipid based formulation that allows the delivery of proteins,

peptides or other bioactive molecules into a broad range of cell types. It interacts non-covalently with the protein creating a protective vehicle for immediate delivery into cells. It fuses directly with the plasma membrane of the target cell. The extent of introduction can be monitored by TRITC-conjugated antibody uptake during hybridization. This is easily monitored using low light fluorescence on living cells. Molecules that have been successfully introduced into various cell types include high and low molecular weight dextran sulfate, B-galactosidase, caspase 3, caspase 8, granzyme B and fluorescent antibody complexes.

2. Electroporation of plasma membrane, a technique commonly used for introduction of foreign DNA during cell transfections, can also be used. This method introduces large size, temporary openings in the plasma membrane, which allows free diffusion of extracellular components into cells.

**[0028]** The methods of the present invention can be used to effect de-differentiation and re-differentiation of any type of germ cell or somatic cell. Examples of cells that may be used include but are not limited to fibroblasts, B cells, T cells, dendritic cells, keratinocytes, adipose cells, epithelial cells, epidermal cells, chondrocytes, cumulus cells, neural cells, glial cells, astrocytes, cardiac cells, esophageal cells, muscle cells, melanocytes, hematopoietic cells, osteocytes, macrophages, monocytes, and mononuclear cells.

**[0029]** The cells with which the methods of the invention can be used can be of any animal species; e.g., mammals, avians, reptiles, fish, and amphibians. Examples of mammalian cells that can be de-differentiated and re-differentiated by the present invention include but are not limited to human and non-human primate cells, ungulate cells, rodent cells, and lagomorph cells. Primate cells with which the invention may be performed include but are not limited to cells of humans, chimpanzees, baboons, cynomolgus monkeys, and any other New or Old World monkeys. Ungulate cells with which the invention may be performed include but are not limited to cells of bovines, porcines, ovines, caprines, equines, buffalo and bison. Rodent cells with which the invention may be performed include but are not limited to mouse, rat, guinea pig, hamster and

gerbil cells. Rabbit cells are an example of cells of a lagomorph species with which the invention may be performed.

**[0030]** Specific somatic cells with which the invention can be performed are human skin fibroblasts transgenic for mouse Oct4 promoter-driven GFP gene. The mouse Oct4 promoter can drive GFP expression in porcine and bovine preimplantation embryos (Kirchhof, et al., 2000). Oct4 is the only known molecular marker of pluripotency that has been shown to be absolutely required for normal development of pluripotent mammalian inner cell mass during early embryogenesis. Pluripotent embryos and embryonic stem cells as well as embryonic-derived tumors are the only tissues in mammals that show expression of this gene (Schöler et al., 1991, Pesce and Schöler, 2000). For example, the mouse Oct4 promoter and its regulatory 5'UTR (8 Kb - H. Schöler) can be used to direct expression of GFP gene as a marker of successfully de-differentiated cells.

#### Introducing extract using BioPorter<sup>®</sup> reagent

**[0031]** Donor somatic cells can be grown as monolayers in tissue culture dishes and synchronized in G1 phase of the cell cycle by methods described in literature (Leno et al., 1992). For example, growing primary cultures can be synchronized by an initial S phase block for 20 hours with 2.5 mM thymidine, followed after a 5 hour interval by a 9 hour mitotic block by demecolcine. Three hours after release from demecolcine, the cells synchronously enter G1 phase. BioPorter<sup>®</sup> reagent coated cell extract can be added to the cultured cells and incubated 4 hours at 37°C. The cells that incorporated extract can be identified and separated from the other cells, e.g., by washing and sorting them using fluorescence assisted flow cytometry (FACS) with detection of the presence of the TRITC-labeled control immunoglobulin in cells. Positive, fluorescent cells can be collected, the medium replaced with stem cell medium, and the cells cultured using conditions designed for stem cells.

Introducing extract using electroporation

**[0032]** Alternatively, the extracts can be electroporated into the target cells; e.g., using methods developed for hybridoma formation. The electroporation procedure introduces holes in the plasma membrane that permit entry of large protein extracellular molecules into cells without the requirement for an active uptake. Electroporation parameters are tested and optimized for the specific donor cell type.

**[0033]** As stated above, the extent of delivery can be monitored by the presence of TRITC-conjugated antibody inside the donor cells after the 4-hour hybridization period. Optimal parameters, e.g., concentrations of BioPorter®, the cell extract, and duration of treatments, can be determined experimentally in order to achieve 50% uptake. Uptake can be monitored by live time-lapse video imaging on an inverted microscope, equipped with an environmental chamber. TRITC-positive cells can be separated from non-positive cells by flow cytometry and used for putative stem cell culture. The expression of Oct4-GFP in live cells can be measured to evaluate the timing and progress of the de-differentiation process occurring within the treated cells.

**[0034]** The proportions of cells that take up extract may exceed 50% using either electroporation or BioPorter® system. Different donor cell types may require unique electroporation and/or BioPorter® conditions; these can be determined experimentally. The procedure can introduce amounts of reprogramming matrix sufficient to effect de-differentiation into the majority of manipulated cells; consequently high numbers of putative stem cells can be obtained in each experiment. The introduced reprogramming matrix is retained by the cells regardless of the method by which it is introduced. Activity of the reprogramming matrix lasts at least 48 hours after hybridization. During this time cells can be kept in a maintenance medium that prevents growth and DNA replication in order to extend the duration of G1 reprogramming phase. Cells synchronized in G1 will be most likely affected by the matrix and the most likely to revert into stem cells (Campbell et al., 1996). After reprogramming, the cells re-enter the cell cycle, retain TRITC fluorescence (indicative of non-leakage) and continue cycling in a manner representative of stem cells. At the time of

de-differentiation GFP positive (green) cells are observed, and FACS will separate the GFP positive cells from the rest.

**[0035]** The efficiency of delivery using the BioPorter<sup>®</sup> system depends on the cells' density and/or confluence, delivery time, amount of protein in the extract to be delivered, concentration of the protein solution during preparation of the complexes (BioPorter<sup>®</sup>-protein complexes) and the hydration volume for BioPorter<sup>®</sup> reagent. Accordingly, these parameters are can adjusted and the protocol optimized for delivery into 50% or more of the target cells. If protein concentration of the cytoplasmic extract is determined to be too low, the extracts can be lyophilized and the concentration of proteins optimized by dry weight. The fraction of the lysate or a combination of 2 or more fractions that is/are responsible for the reprogramming can be identified by HPLC fractionation of the extract and testing of the fractions individually for their reprogramming ability. The invention includes identifying and using those fraction(s) of the whole extract that are required to effect active reprogramming (de-differentiation).

**[0036]** Different donor cell types are likely to require different amounts of active extract and/or different duration of delivery in order to de-differentiate. Accordingly, different somatic cell types can be examined for their susceptibility for reprogramming, e.g. skin fibroblasts, keratinocytes, hair follicle cells, white blood cells and muscle cells. Upon demonstration that a certain cell type is particularly amenable to reprogramming, that cell type can then be used in subsequent experiments. Cell extracts obtained from oocytes, teratocarcinoma cells and spermatogonia are expected to display different reprogramming capacity. Their reprogramming capacity will be correlated with the ease of preparation, ability to generate sufficient volumes and protein quantity, repeatability of preparation, consistency of reprogramming activity and ease of delivery. Optimizing these factors is within the level of skill in the art.

**[0037]** In addition to BioPorter<sup>®</sup> and electroporation, reprogramming extracts can be introduced into cells using membrane enclosed cytoplasmic fragments from the pluripotent cell types mentioned above; by hybridizing them with donor cells by electrofusion or PEG-mediated fusion.

### Evaluating de-differentiated cells

**[0038]** Embryonic stem cells retain their pluripotency in vitro when maintained on inactivated fetal fibroblasts in culture. More recently, it has been reported that human embryonic stem cells can successfully be propagated on Matrigel in a medium conditioned by mouse fetal fibroblasts (Xu et al., 2001). Human stem cells can be grown in culture for extended period of time (reviewed by Thomson and Marshall, 1998) and remain undifferentiated under specific culture conditions. De-differentiated cells are expected to display many of the same requirements as pluripotent stem cells and can be cultured under conditions used for embryonic stem cells.

Methods for evaluating de-differentiated cells include:

**[0039]** 1. Monitoring changes in the cells' phenotype and characterizing their gene and protein expression. Live time-lapse video imaging can be used to monitor the uptake of the extracts, changes in cell morphology upon hybridization (or lack thereof), and dynamics of changes induced as well as GFP transgene fluorescence.

**[0040]** 2. Screening results can be compared to results obtained with undifferentiated, pluripotent control cells such as monkey parthenogenetic stem cells (Advanced cell technology), or human embryonic stem cells (Wisconsin Alumni Research Foundation, Madison, WI and Geron, Inc). Stem cell markers and morphometric and growth characteristics of parthenogenetic cynomolgous monkey embryonic stem cells (Cibelli et al., Nature, in press) match with those published by Thomson et al. (1998) for human embryonic stem cells obtained from in vitro fertilized human blastocyst.

**[0041]** The expression of the following genes of de-differentiated cells and human embryonic stem-like cells can be compared: alkaline phosphatase, Oct4, SSEA-3, SSEA-4, TR-1-60 and TR-1-81 (Thomson et al., 1995, 1998). Assays designed to detect expression of genes specific to the given cell type can be used to confirm the presence of expression in the cells prior to hybridization, and to confirm the absence of expression after hybridization.



Self-renewing capacity, marked by induction of telomerase activity, is another characteristic of stem cells that can be monitored in de-differentiating cells (Morrison et al., 1996).

#### Maintenance of the undifferentiated state

**[0042]** Mouse fetal fibroblasts can be mitotically inactivated by irradiation and prepared at  $5 \times 10^4$  cells/cm<sup>2</sup> on tissue culture plastic previously treated by overnight incubation with 0.1% gelatin (Robertson, 1987). Fibroblasts can be prepared a day before hybridization construction and cultured in DMEM, supplemented with 20% fetal bovine serum, 0.1 mM mercaptoethanol and 0.1 mM non-essential amino acids and human recombinant LIF.

As an additional means to maintain an undifferentiated state, hybrid cells growing on fibroblast feeder layers, can be supplemented with GCT44 factor (human yolk sac teratoma cell factor; Roach et al., 1993). Gene expression can be determined by RT-PCR, and translation products by immunocytochemistry and Western blotting. Markers for the expression of specific genes in the donor cells can be identified depending on the cell type. For example, the fibroblast surface protein gene can be used as a marker for expression in fibroblasts, etc. RT-PCR assays can be used to demonstrate expression in donor cells and absence of the product is an indication that expression of that gene has been lost. To evaluate de-differentiation, induction of expression of SSEA-3, SSEA-4, TR-1-60, TRA-1-81, alkaline phosphatase and Oct4 can be monitored. Immunocytochemistry can be used to detect gene products. RT-PCR primers and hybridization probes and antibodies for immunocytochemistry and Western blotting are commercially available. Expression of Oct4GFP transgene can be monitored by live fluorescence microscopy.

**[0043]** Telomerase activity is assayed as described by Thompson et al. (1998). The TRAPEZE telomerase detection kit is used (Oncor, Gaithersburg, MD). About 2000 cells are analyzed at every experimental time point and 800 cell equivalents are loaded in each well of a 12.5% nondenaturing polyacrylamide gel. Reactions are done in duplicates. Finally, cells can be injected into SCID mice and monitored for development of teratomas. After 6

weeks, teratomas are analyzed by histological sectioning and presence of various tissues determined. Assay can also be performed to determine the potential of the cells to induce formation of embryoid bodies and to undergo spontaneous differentiation in culture.

**[0044]** Temporal expression of key marker genes can be monitored at each passage to determine the timing of reprogramming in the hybridized cells. This yields information as to how long it takes for the somatic cell (differentiated state) to de-differentiate with respect to its gene expression profile. Morphology of de-differentiated cells, timing and progression of cell cycles and doubling times can be monitored daily by live time-lapse video imaging in parallel with incubated cultures. In addition, mitotic cells can be shaken off the monolayers and used for gene expression analysis and ICC after different numbers of passages. Their gene expression profile is compared with that of the somatic donor cell type. The length of time de-differentiated cells can be maintained in culture is monitored and any change in morphology or gene expression determined. Observation that the hybridized cells display loss of tissue specific protein and gene markers, display change in morphology and acquire stem cell markers is evidence that the cells have undergone de- differentiation and are suitable for induced differentiation.

**[0045]** De-differentiated cells may be slow cycling, with the majority of the cells in G1 phase of the cell cycle, they may display higher nucleocytoplasmic ratio than donor somatic cells, possess poor rhodamine uptake into mitochondria, display telomerase activity that is higher than that in untreated cells; and they will express Oct4-GFP. Different donor cell types may demonstrate a variable ability to revert their nuclear function. Growth requirements are generally similar to those of parthenogenetic stem cells, and so is protein and gene expression. Different extracts may induce various degrees of reprogramming. Oocyte extracts are more likely to induce a change into embryonic-like stem cells, while teratocarcinoma and spermatogonial extracts may be more limiting in their ability to reprogram the cells completely.

**[0046]** Partial if not complete reprogramming can occur within the first 24-48 hours after matrix delivery. The extent of reprogramming depends on the

donor cell type, cell cycle stage of donor cells, and extract quality/fraction. Tissues originating from different germ layers may have different ability to undergo reprogramming. Expression of pluripotent markers is expected to continue as long as the hybridized cells are cultured under conditions that will maintain their undifferentiated state. Similarly, telomerase activity is expected to be detectable in de-differentiated cells, evidence that the cells have acquired self-renewing capacity.

#### A differentiation protocol for pancreatic islets

**[0047]** Pancreatic cells have been reportedly detected at a low frequency in mixed cell populations derived from induced differentiation of embryonic stem cells (Kahan et al., 2001, Schuldiner et al., 2001). The present invention provides a new approach for inducing and directing pancreatic differentiation.

**[0048]** Directed differentiation of stem cells into endoderm-derived cell lineages has not been describe. Except for the demonstration that NGF and HGF (Schuldiner et al., 2000) induce transcription of some endodermal markers (such as albumin, alpha-feto protein, amylase and alpha 1AT) in addition to markers for ecto- and mesodermal development, there is no published literature on directed endoderm differentiation. Lumelsky et al. (2001) reported in Science that they successfully achieved differentiation of mouse embryonic stem cells into endocrine pancreatic, insulin-secreting cells in vitro by first growing mouse embryonic stem cells into embryonic bodies. This is the first time that a significant proportion of stem cells have been reported to actually follow insulin positive differentiation (35% of all stem cells).

**[0049]** Lateral mesoderm (hematopoietic cells) can transdifferentiate into endoderm (liver cells; Theise et al., 2000); accordingly, pancreatic development is expected to occur in a two-step process.

**[0050]** Cell differentiation is defined and supported by the cell's environment; therefore, it is possible to design extracellular matrix, media and supplement combinations that induce pancreatic development. Bovine fetal pancreatic primary cultures (both monolayers and suspension cultures) as "feeders" for stem cell differentiation and pancreatic extracts as supplements to

differentiation medium can be used as substrates/helpers for induced differentiation.

**[0051]** Long-term survival and stability of physiological responses has been afforded only by extracts enriched in extracellular matrix. Matrigel (Brill et al., 1994; Grant et al., 1992) has induced cells into far more complicated physiological states than any known purified matrix component by itself. A major function of the matrix is to allow for assembly of cells into a three-dimensional structure, which is essential for achieving fully normal phenotype and for normal transcription rates of tissue-specific genes (Rodriguez-Boulan and Zorzolo, 1993). Extracellular lateral and basal matrix components can be combined to achieve the most physiological conditions for pancreatic development. Cell adhesion molecules (CAMs), proteoglycans (lateral matrix between the same type of cells), laminin and type IV collagen can be provided as components of basal matrix. Extracellular (EC) matrix can be used in combination with a nutrient rich medium, supplemented with fetal bovine pancreas extract and/or supplemented with bovine fetal pancreatic cells embedded in porous gelatin matrix sandwich. Optimal concentrations of HDL/LDL-high and low density lipoproteins, PL-phospholipids, FFA-free fatty acids, bFGF, heparin proteoglycans and glucocorticoids can be determined by routine assays. Pancreatic extracts are prepared using similar methods as for reprogramming matrix extracts.

**[0052]** Flow cytometric sorting strategies can be developed based on the developing and mature surface antigenic profiles of pancreatic cells. Cells are separated using stem cell surface antibodies to eliminate non-committed cells. Serum-free hormone defined medium (HDM) is used instead of animal serum for all culture in order to allow for reproducibility.

Developing cultures are grown on an inverted microscope in an environmentally controlled chamber and a parallel control in a low oxygen incubator. At regular intervals, images are recorded using live, time-lapse video imaging system (in house) and processed to determine change in morphology and population doubling time.

**[0053]** Imaging data obtained is analyzed by Metamorph (Universal Imaging,

PA) and real-time developmental sequence reconstructed for analysis. Cells can be sampled every 24-48 hours for immuno-cytochemistry. They can be spun onto glass slides using Cytospin centrifuge (in house) and assayed for loss of stem cell markers as well as acquisition of endodermal and pancreatic markers, such as insulin I and II, glucagon, PDX-1 transcription factor, somatostatin, alpha-amylase, anti-islet amyloid polypeptide-IAPP, glucose transporter 2, and carboxypeptidase A (Chemicon, Temecula, CA and BabCo, Richmond, CA). The same samples can be analyzed the presence of specific mRNAs by RT-PCR, and for determination of telomerase activity. Presence of insulin in the cells is detected by dithizone (DTZ) staining (Ricordi et al., 1994). Briefly, 10 mg of DZT is dissolved in 1 ml of DMSO (10 mg/ml stock) and 0.5 mg/ml final solution for labeling made in tissue culture medium, supplemented with 2% FCS. Cells are labeled and red staining indicates presence of insulin. Insulin positive cell are counted followed by determination of the percentage of insulin-positive cells in the total cell population.

**[0054]** Various cell types are generated using the above protocol, however, induction of endoderm-derived cell types is significantly enriched when compared to default differentiation from embryonic bodies (Lumelsky et al., 2001). All three types: endocrine, exocrine and ductal cell types can develop, as a complex, three-dimensional substrate will be provided.

**[0055]** Genes that have been implicated in early determination of pancreatic endocrine lineages include basic-Helix-Loop-Helix (bHLH) transcription factors (Isl1, Nkx2.2, NeuroD/B2, Pax4 and Pax 6; Sander and German, 1997; Edlund, 1998, 1999; St-Ogne et al., 1999) and the PDX1 homeobox gene. If necessary, constructs can be designed with promoters of these genes driving a GFP reporter, and a neomycin trap. Transgenic cells would allow for not only monitoring of cells for expected gene expression but also allow for selection of transgenic cells actively transcribing pancreatic genes to be selected for by neomycin supplemented medium. It is interesting that the same genes are expressed during early neuronal development, which suggests that early development of several tissues may be under similar control. The initiation of pancreatic development and cell-type specification are two of the three levels of

development that can be accomplished. The third one (progression of pancreatic development) determines organogenesis and is not anticipated. Initiation is monitored by detection of a beta-cell-specific Hb9 homeobox gene and Isl1/PDX1 gene expression (Odorico et al., 2001). For specification of cell fate, ngn3 gene expression is monitored.

Maintaining stable morphology and function of newly differentiated cells

**[0056]** It is anticipated that cultures of pancreatic cell can be used for transplantation immediately or cryopreserved for later use. It is important to examine cell functionality and lifespan in vitro prior to initiating transplantation studies in mice. Cultures of primary pancreatic cells have been described and we have been successful in culturing fetal bovine pancreatic cells for over 2 months. Cells retain their morphology, remain non-adherent, display classic endocrine morphology with large cytoplasmic vesicles and form colonies indicative of pancreatic islets. They can be subcultured and are well supported without extracellular matrix when grown in hepatocytes (HGM) and endothelial growth media (EGM; both are serum-free; Dominko et al., unpublished). Newly developed pancreatic cells are cultured using the same conditions.

**[0057]** Pancreatic cells are grown at low density in suspension using EGF and HGF media. The cells are sampled at regular intervals and assayed for maintenance of insulin synthesis. At every third to fourth passage, the cells are examined by ICC for continued presence of pancreatic markers, for karyotype stability and telomerase activity. Islets are evaluated by criteria proposed by Ricordi et al. (1994).

**[0058]** Using the de-differentiation methods of the present invention, pancreatic cells can be generated from non-transfected, de-differentiated cells to avoid introducing transgenes into a potentially therapeutic cell population. Alternatively, transgenic donor cells may be used; e.g., to trace the cells during animal testing.

**[0059]** Endocrine pancreatic cells are expected to retain their morphology and function for at least 2 months in culture. Due to their relatively slow growth, we expect telomerase to remain active for extended periods of time and

karyotype should remain stable at 2n. However, to alleviate any potential difficulties, pancreatic islets are transplanted into diabetic mice as soon as sufficient cell numbers are available.

#### Testing functionality of pancreatic islets by transplantation

**[0060]** For human islets, attempts have been made to ascertain islet viability in vivo by transplantation into nude (SCID) rodents, to avoid rejection. These animals have a deficient immune system due to congenital thymic aplasia and are unable to reject transplanted xenogenic tissue. The first report of transplantation dates to 1974 (Povlsen et al.). Several portions of human fetal pancreas were transplanted subcutaneously and histological examination of the excised tissue two months after transplantation revealed a relatively normal lobular appearance with no sign of rejection. Subsequently, a number of groups reported further success with transplantation of human fetal pancreatic tissue and isolated islets into SCID mice (Ricordi et al., 1988, 1991), made diabetic with streptozotocin. Long-term graft survival and functionality were demonstrated. Upon surgical graft removal, mice returned to a diabetic state

**[0061]** Animal experimental protocol has been submitted to the Institutional Animal Care and Use Committee (IACUC) and we expect the protocol to be approved by July 2001. Experimental diabetes will be induced in 10-12 week old male 12/sv mice by a single intraperitoneal injection of streptozotocin (120-150 mg/kg of body weight) in citrate phosphate buffer; pH 4.5; Sigma Chemical Co. St. Louis, MO) (Soria et al., 2000). Stable hyperglycemia (300-600 mg/100ml) is expected to develop within 48-72 hours. Blood glucose levels will be determined using a blood glucose analyzer (Glucometer Elite XL, Bayer Corp., Elkhart, IN). The animals will be grafted with cells or with a buffer vehicle 24-48 hours after the establishment of stable hyperglycemia.  $1-2 \times 10^6$  cells in suspension will be injected per animal under the kidney capsule.

#### Data Collection and Analysis:

**[0062]** Glucose levels can be monitored every 24 hours after grafting. Each transplanted animal serves as its own control, since it is possible to perform

nephrectomy of the kidney bearing the graft and produce a rapid return to the diabetic state. In addition, histological studies of the renal subcapsular grafts provide information on the morphologic integrity and cellular composition of the transplanted islets at the end of the study (52 weeks). Data can be analyzed by 2-way ANOVA (accounting for cell line effect and animal effect) and difference in glucose levels evaluated at  $P=0.05$ .

**[0063]** Return to normal glucose levels is expected to occur between two and three weeks after transplantation if the islets retain their functionality (Buschard et al., 1976). Graft function is expected to persist for at least a year (Tuch et al., 1984).

### EXAMPLES

Example 1 Preparation of high-speed metaphase II *Xenopus* oocyte extract.

**[0064]** Mature *Xenopus laevis* females were superovulated with PMSG and 72 hours later induced to ovulate with hCG. Eggs were collected in cold MMR buffer (100 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 5 mM Hepes, see Julian Blow, 1993) and washed 2 times with High Salt Barth Solution (NaCl 110 mM, Tris-HCl 15 mM, KCl 2 mM, NaHCO<sub>3</sub> 2 mM, MgSO<sub>4</sub> 1 mM, Na<sub>2</sub>HPO<sub>4</sub> 0.5 mM), EGTA 2 mM). The jelly coats were removed with cold 2% L-cystein free base (Sigma) with 2 mM EGTA at pH 7.8 (adjusted with 6N NaOH). Eggs were washed in unactivating extraction buffer (KCl 50 mM, Hepes 50 mM, MgCl<sub>2</sub> 5 mM, EGTA 5 mM, Beta-mercaptoethanol 2 mM), and were packaged into 4.4 ml Sorvall® tubes. Excess buffer was removed, and the eggs were crushed by centrifugation in a swinging bucket rotor at 10,000 rpms for 15 minutes. The cloudy, gray middle cytoplasmic layer was removed and centrifuged at 20,000 rpm for 15 min at 4°C. The translucent layer was removed and diluted 1:6 with extract dilution buffer at 4°C (KCl 50 mM, Hepes 50 mM, MgCl<sub>2</sub> 0.4 mM, EGTA 0.4 mM; supplemented just before use with DTT 2 mM, 10 ug/ml aprotinin, leupeptin and cytochalasin B each). The extract was diluted 1:6 with the extract dilution buffer. The extracts were centrifuged again at 30,000 rpm for 1.5 hours at 4°C. Two layers were removed: a translucent layer and a golden



layer. These were aliquoted at 50 µl/vial, snap frozen in LN2 and stored at -80 C.

Example 2 Preparation of metaphase II stage bovine oocyte extract

**[0065]** Mature bovine oocytes were aspirated from freshly collected ovaries and were matured in vitro. The oocytes were collected at 20 hours post maturation and stripped free of surrounding cumulus cells by vortexing in 2.5 mg/ml hyaluronidase (Calbiochem) dissolved in DPBS (Biowittaker). Zonae were removed by incubation in 0.5% w/v pronase (Calbiochem) dissolved in DPBS (Biowittaker) and zona-free oocytes washed through several washes of manipulation medium (Modified ACM, designated ACM-P). The oocytes were resuspended in a small amount of fusion medium (200 oocytes in 20 µl of 0.28 M mannitol, 50 µM MgCl<sub>2</sub>, 0.1 mg/ml PVP 40 kD, all Calbiochem) and vortexed at high speed for 3 minutes. The vortexed material was examined under a stereomicroscope to confirm the absence of membrane-enclosed cytoplasmic fragments. Oocyte lysate was prepared freshly for each use and kept on ice until use.

Example 3 Preparation of bovine adult skin fibroblasts

**[0066]** Tissue samples from 2 mm circular ear punch biopsies were received in transport media made of DPBS (Biowittaker) supplemented with Ciproflaxin® (Mediatech, Cat#61-277-RF). A tissue sample was removed from the container using sterile technique, and placed into a 60 mm falcon petri dish with IMDM (Gibco) and zonkers, fungizone, and pen/strep and allowed to soak for 10 minutes. Using a dissecting microscope, the excess connective tissue was removed and the remaining skin moved to another 60 mm petri dish with above medium. The sample was then placed into a 60 mm petri with about 2 ml of medium and minced into small pieces. Fresh medium was added to the dish to loosen the pieces, then all contents added to a T25 tissue culture flask and the final volume of medium was brought to 3 ml. The sample was incubated at 38.5° C in 5% CO<sub>2</sub> in humidified air for 10 days without changing medium or moving the flask. The cells were then passaged, first to a T75 flask using Trypsin-EDTA (Gibco), then to 4 T75 flasks, and then were frozen in

complete medium with 10% DMSO. Prior to use, cells were thawed at 37 C and centrifuged at 800 xg for 4 minutes to remove the cryoprotectant and seeded into a 100 mm culture dish 24-48 hours prior to use. Prior to electroporation, cells were trypsinized and washed in culture medium by centrifugation and suspended in culture medium without serum.

Example 4 Electroporation of Xenopus oocyte extract into adult bovine skin fibroblasts.

**[0067]** Proliferating bovine adult skin fibroblasts growing on 100 mm tissue culture dishes at about 90% confluence (Figure 1) were harvested using a 1:1 dilution of trypsin-EDTA (Gibco, Cat# 15400-096) in DPBS without calcium and magnesium. The cells were pelleted by centrifugation and resuspended in fusion medium at  $1.0 \times 10^6$  per ml. Twenty  $\mu$ l of cell suspension was added to 20  $\mu$ l of oocyte lysate and mixed. The cell-lysate mixture was transferred to a 0.5 mm gap width platinum wire electrofusion chamber (BTX Model # 450-1) and electroporation was achieved using 2 consecutive DC pulses of 2.0 kV/cm for 15  $\mu$ sec each. Control experiments were conducted where the oocyte lysate was loaded with 10  $\mu$ M Cytotracker Blue (Molecular Probes) membrane impermeable cell tracking dye for 45 minutes and washed for 30 minutes. Observation of surviving cells 2 hours after electroporation using fluorescence microscopy confirmed the presence of tracking dye inside the cells, indicating successful transfer of extracellular material into the cells during the electroporation process. Following electroporation, cells were transferred to 1 ml of holding medium (ACM-P) and incubated for 30 minutes at 37 C. Cells were concentrated by centrifugation at 800 x g for 4 minutes and transferred to 50  $\mu$ l drops of KSOM (Cell and Molecular Technologies) in 35 mm petri dishes (Falcon) covered with mineral oil (JT Baker). Cultivation and characterization of bovine adult fibroblasts electroporated with high speed xenopus oocyte extract was as follows:

**[0068]** Within 4 days after electroporation, the cells formed colonies morphologically similar to embryonic stem cell colonies (Figure 2). Cells surrounding the ES-like colonies had an epithelial cell morphological

appearance that was different than that of the fibroblasts used as starting material. Attempt to pass these colonies using standard trypsinization procedures failed, which suggests that biochemical changes to the cell's secretion of extracellular matrix had changed as well. Therefore, the colonies were cut into small clumps of cells using a 27 gauge hypodermic needle (Becton Dickinson). Clumps of cells were plated either onto  $\gamma$ -irradiated E14 mouse embryonic fibroblast feeder cells or onto tissue culture plastic without feeder cells. The culture medium was ES cell medium (DMEM, etc., 15% heat inactivated fetal bovine serum, 1% non-essential amino acids, 0.1 mM  $\beta$ -mercaptoethanol, 100 units/ml PennStrep).

**[0069]** After 7 days, the cells plated on feeder cells failed to proliferate further and were lost upon subsequent subculture, likely due to a poor quality preparation of feeder cells. The cells plated on tissue culture plastic were subcultured into a 100 mm tissue culture dish using a serum-free medium consisting of a 1:1 mixture of DMEM (Gibco) and Ham's F12 nutrient mixture supplemented with Insulin, Transferrin and Selenium (ITS, Gibco). The cells expanded to about 70% confluence and acquired a flattened phenotype and ceased proliferation in this medium. The medium was changed 2x weekly and the cells maintained for 4 weeks. Some of the cells began to display a neuronal phenotype with a "phase bright" appearance of the cell body (Figure 3, A & B). The cells were trypsinized and some were re-plated in 24 well plates at about 70% confluence. Three days later, the cells were fixed with 4% paraformaldehyde in DPBS for analysis of cell type-specific markers by immunocytochemistry.

**[0070]** The remaining cells were plated in 3 replicate 60 mm dishes of cells. After 3 days, the medium was changed to 1) DMEM/F12 ITS; 2) DMEM/F12 ITS with 10  $\mu$ g/ml Nerve Growth Factor (NGF, Supplier XXX); and 3) Neurobasal Medium A (NBA, Clonetics) with 10  $\mu$ g/ml NGF. The cells treated with DMEM/F12 ITS alone displayed a phenotype similar to that observed before. Cells in DMEM/F12 ITS with NGF had a larger number of cells with a neuronal phenotype as well as an increase in cells with longer axonal-like processes (Figure 4). In some cases, the processes from adjacent cells appeared to be in

contact with one another (Figure 5). Cells treated with NBA with NGF failed to develop a neuronal phenotype.

Example 5 Electroporation of bovine oocyte extract into bovine fetal fibroblasts.

**[0071]** The lysate was incubated with  $1 \times 10^6$  growing bovine fetal fibroblasts that have been suspended in 40  $\mu$ l of fusion medium. After mixing, the suspension of cells/lysate was electroporated for 1 msec at 2.0 Kvolts, and the electroporated mixture was placed onto mouse inactivated fetal fibroblasts in embryonic stem cell medium. After culture at 37° C, 5% CO<sub>2</sub> in air for 7 days, the cells formed distinct colonies with appearance similar to those of mouse embryonic stem cells. While we have not yet confirmed the presence of any stem cell markers in these cells, their morphology, characteristic colony growth and nuclear-to-cytoplasmic ratio are indicative of putative stem cells.

Example 6

**[0072]** Primary pancreatic cell cultures were established from two pancreata obtained from a day 60 and a day 90 bovine fetus. The tissue was removed under sterile conditions, minced with fine scissors and plated in DMEM (Sigma Chemical Co., St Louis, MO), supplemented with 10% heat-inactivated fetal calf serum (Hyclone). Primary explants were grown for 3 days in 5% CO<sub>2</sub>. Cells were split into two different subcultures. Non-attached cells that maintained a colony appearance and were growing in suspension were passaged into new HGM medium and remained in suspension. The cells that attached during the first three days were trypsinized and subcultured into fresh HGM. These two cell populations remained distinctly different during progressive culture. Non-attached cells continued to proliferate slowly, remained in floating aggregates resembling islets and were viable after over 2 months of culture. Adherent cells displayed different morphology. They clearly formed small clusters, but these clusters were attached to the bottom of the dish and were surrounded by stromal-like fibroblast cells. This demonstrates our ability to maintain pancreatic cultures in vitro.

**[0073]** Our preliminary data demonstrated that introduction of oocyte cytoplasmic lysate into fibroblasts by electroporation induces a change in morphology. Mature bovine oocytes were collected at 20 hours post maturation and stripped free of surrounding cumulus cells by vortexing in 2.5 mg/ml hyaluronidase. Zonae were removed by incubation in 0.5% pronase and zona-free oocytes washed through several washes of medium. The oocytes were resuspended in a small amount of fusion medium (200 oocytes in 20  $\mu$ l of 0.3 M sorbitol, 50  $\mu$ M  $MgCl_2$ ) and vortexed at high speed for 3 minutes. The vortexed material was examined under a stereomicroscope to confirm the absence of membrane-enclosed cytoplasmic fragments. The lysate was incubated with  $1 \times 10^6$  growing bovine fetal fibroblasts that have been suspended in 40  $\mu$ l of fusion medium. After mixing, the suspension of cells/lysate was electroporated for 1 msec at 2.0 Kvolts and electroporated mixture placed onto mouse inactivated fetal fibroblasts in embryonic stem cell medium. After culture at 37°C, 5%  $CO_2$  in air for 7 days, the cells formed distinct colonies with appearance similar to those of mouse embryonic stem cells. While we have not yet confirmed the presence of any stem cell markers in these cells, their morphology, characteristic colony growth and nuclear-to-cytoplasmic ratio are indicative of putative stem cells.

**[0074]** Figure 6 contains the results of this experiment and shows bovine fetal pancreas primary cell culture 3 days after isolation. Cells either plated down (A) or remained in suspension in aggregates (B). Pancreatic cells four weeks after initiation of culture (C). Bovine fibroblast primary cell cultures (controls, D) were dissociated by trypsinization and electroporated with CytoTracker Blue (Molecular Probes, Eugene, OR) prelabeled bovine oocyte lysate. After the electroporation, cells were plated on gelatin coated cell culture dishes and examined for the presence of CytoTracker Blue 24 hours later (E-phase, F-fluorescence using UV excitation). After 1 week in culture, the cells started forming colonies resembling stem cell aggregates (G), which increased in size during the following 2 weeks (H, I). All images were taken at 100X, recorded with DAGE-MTI camera and printed on a UVP printer. Images were scanned into Adobe Photoshop and pseudo-colored.

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What is claimed:

1. A method for effecting de-differentiation of a somatic cell comprising
  - (a) culturing a somatic cell in the absence of growth factors, cytokines, or other differentiation-inducing agents,
  - (b) introducing components of cytoplasm of pluripotent cells into the somatic cell; and
  - (c) allowing the cell to de-differentiate.
2. The method of claim 1, wherein the cell is a mammalian somatic cell selected from the group consisting of fibroblasts, B cells, T cells, dendritic cells, keratinocytes, adipose cells, epithelial cells, epidermal cells, chondrocytes, cumulus cells, neural cells, glial cells, astrocytes, cardiac cells, esophageal cells, muscle cells, melanocytes, hematopoietic cells, macrophages, monocytes, and mononuclear cells.
3. The method of claim 1, wherein step (a) comprises culturing the cell in serum-free medium.
4. The method of claim 1, wherein the pluripotent cells are selected from the group consisting of oocytes, blastomeres, inner cell mass cells, embryonic stem cells, embryonic germ cells, embryos consisting of one or more cells, embryoid body (embryoid) cells, morula-derived cells, teratoma (teratocarcinoma) cells, as well as multipotent partially differentiated embryonic stem cells taken from later in the embryonic development process.
5. The method of claim 1, wherein the pluripotent cells are oocytes.
6. The method of claim 5, wherein the oocytes are metaphase II oocytes.
7. The method of claim 5, wherein the oocytes are *Xenopus* oocytes.
8. The method of claim 1, further comprising centrifuging oocyte cytoplasm, and isolating a fraction of the centrifuged oocyte cytoplasm containing the components of cytoplasm of step (b).
9. The method of claim 1, wherein step (b) comprises placing the somatic cell in solution containing components of cytoplasm of pluripotent cells, and introducing components of cytoplasm of pluripotent cells into the somatic cell by electroporation.



10. The method of claim 1, further comprising, after the step of introducing components of cytoplasm of pluripotent cells,  
culturing the cell under conditions suitable for maintaining pluripotent stem cells in an undifferentiated state
11. The method of claim 1, further comprising, after the step of introducing components of cytoplasm of pluripotent cells,  
culturing the cell under conditions that induce or direct partial or complete differentiation to a particular cell type
12. The method of claim 12, comprising, after the step of introducing components of cytoplasm of pluripotent cells,  
culturing the cell in medium containing nerve growth factor.
13. The method of claim 12, comprising, after the step of introducing components of cytoplasm of pluripotent cells,  
culturing the cell in DMEM/F12 ITS medium that contains nerve growth factor.
14. A method for reprogramming a somatic cell to become a cell of neural lineage, comprising:
  - (a) culturing a somatic cell that is not of neural lineage in the absence of growth factors, cytokines, or other differentiation-inducing agents,
  - (b) introducing cytoplasm of a pluripotent cell into the cell; and
  - (c) culturing the cell in medium containing nerve growth factor.
15. The method of claim 14, wherein step (c) comprises culturing the cell in DMEM/F12 ITS medium that contains nerve growth factor.
16. The method of claim 14, further comprising assaying to detect a marker of cells of neural lineage.
17. A composition of cells of neural lineage prepared by the method of claim 14.



Figure 1



Figure 2

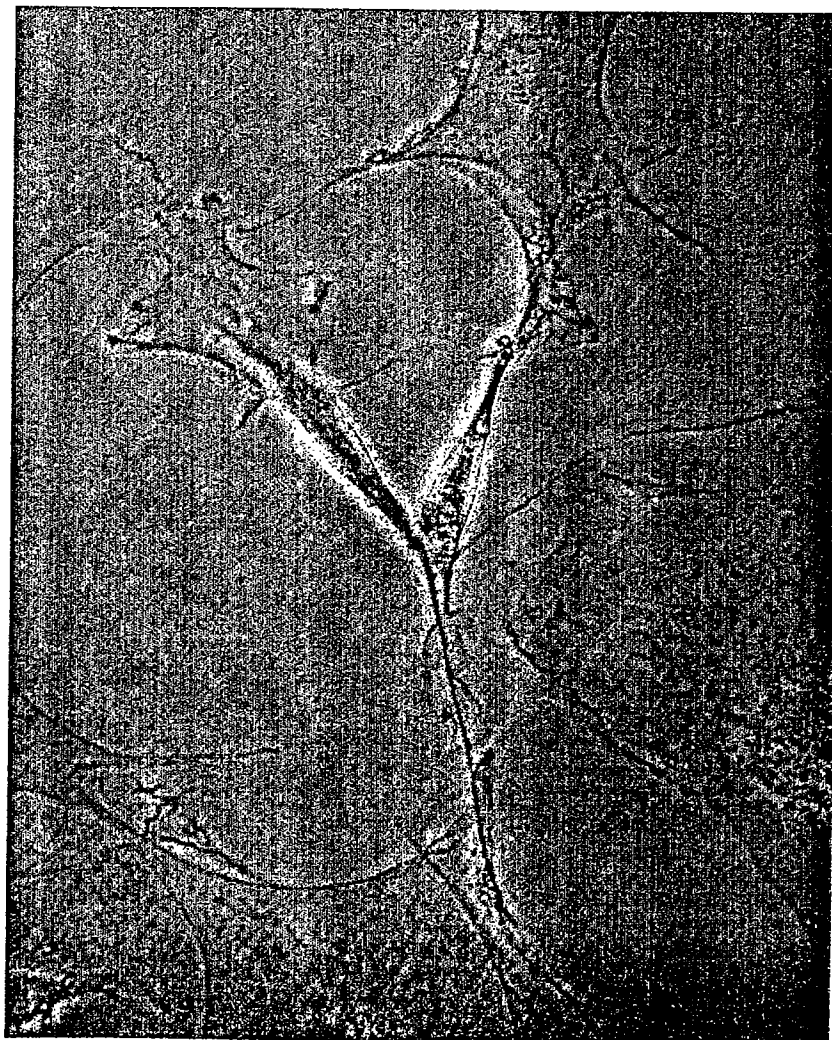


Figure 3A

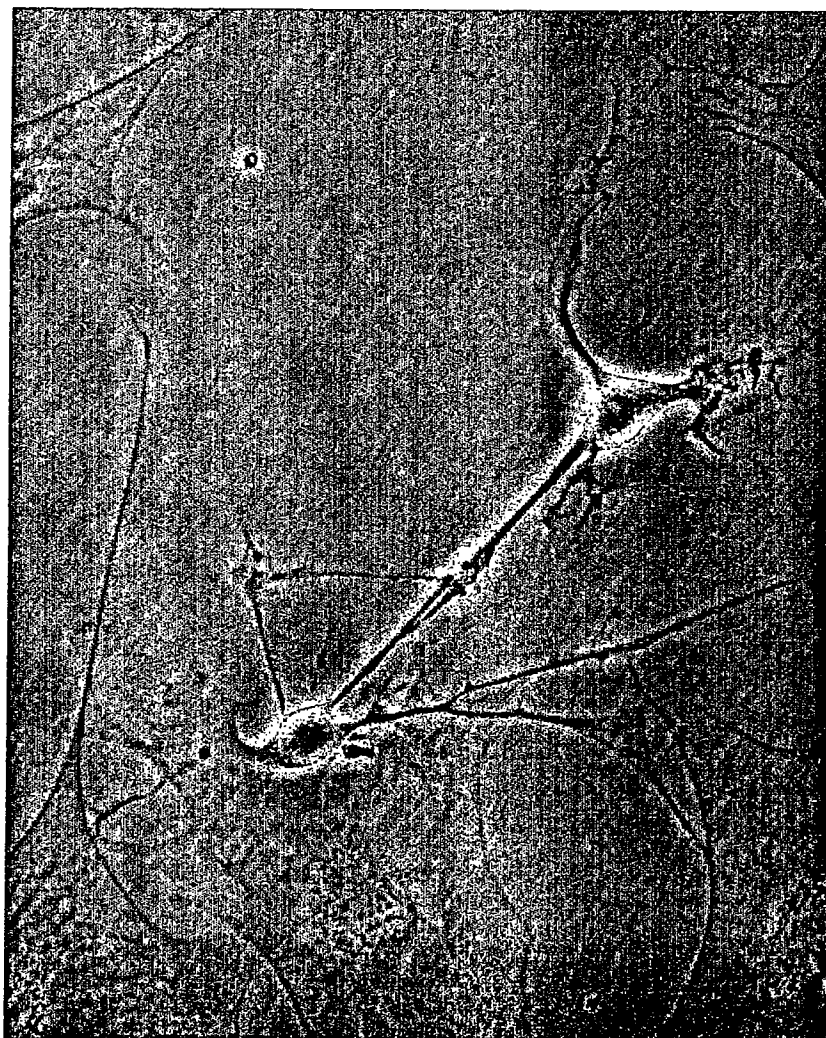


Figure 3B

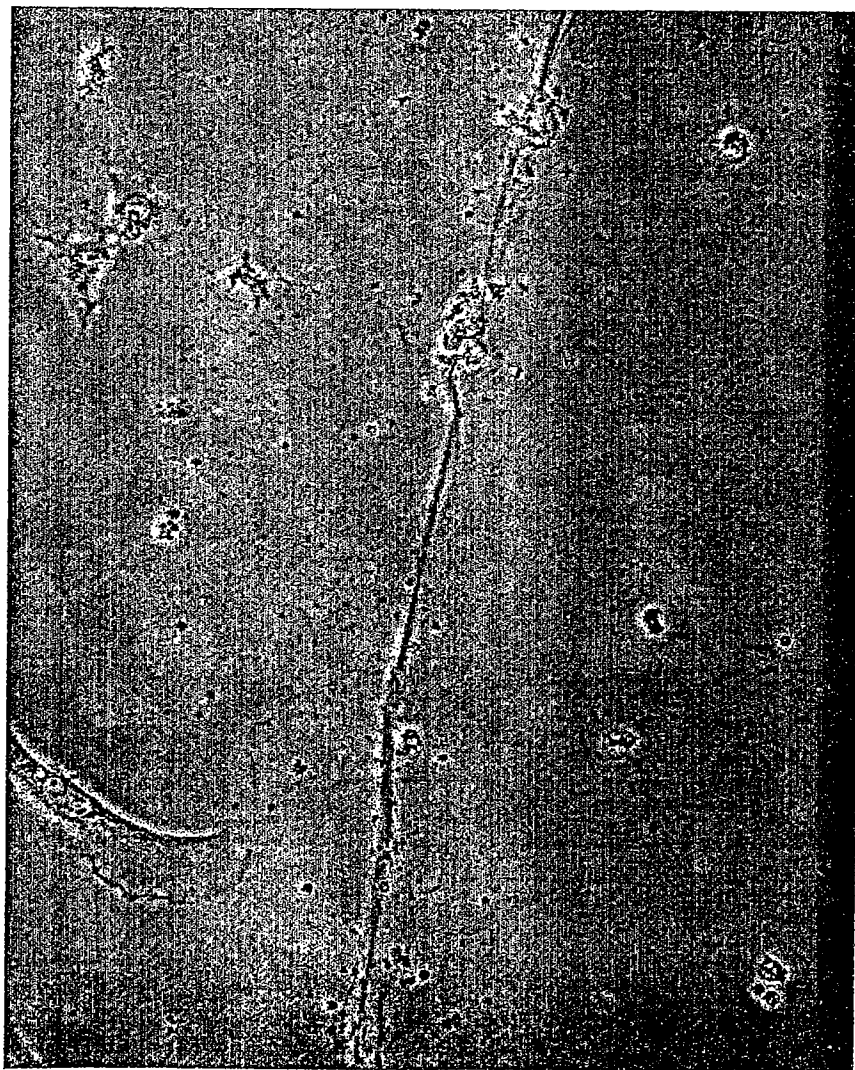


Figure 4

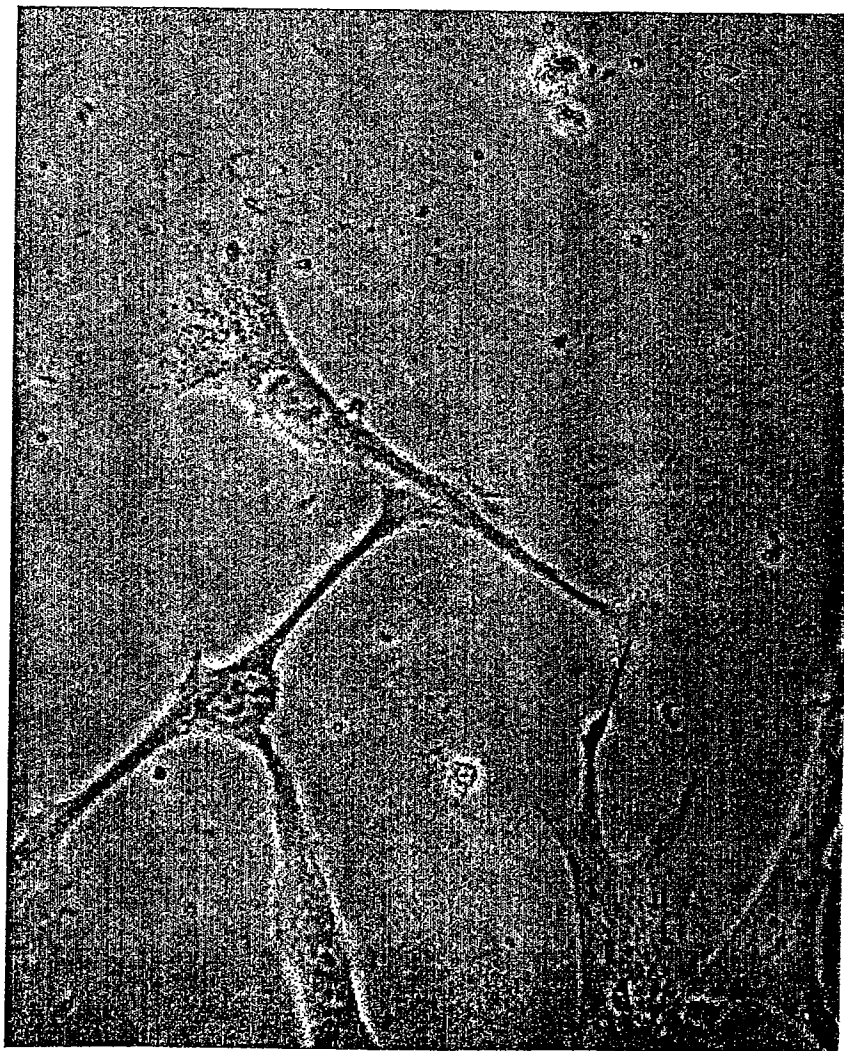


Figure 5

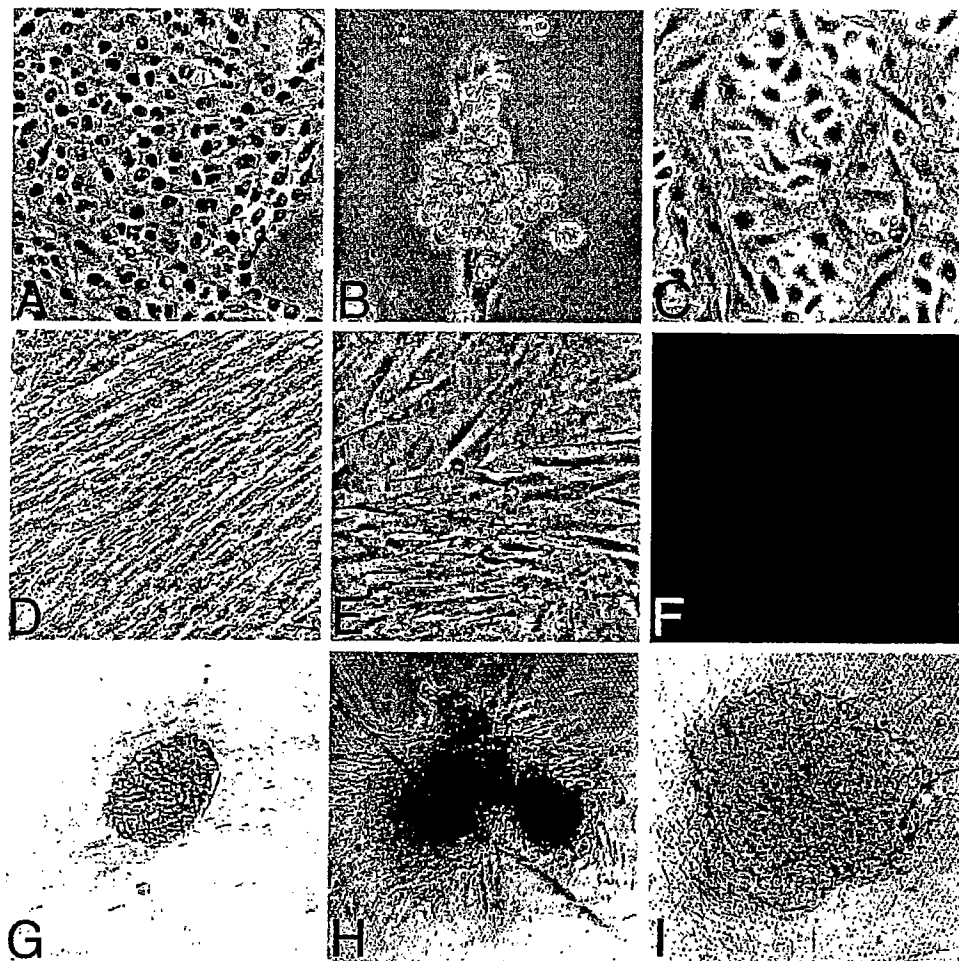


Figure 6



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/26798

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) : C12N 5/00  
 US CL : 435/325, 375, 377

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
 U.S. : 435/325, 375, 377

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
 EAST CAPLUS BIOSIS EMBASE

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	COLLAS, P. Cytoplasmic Control of Nuclear Assembly. Reproduction, Fertility and Development. 1998, Vol. 10, pages 581-592.	1-16
A	KATAGIRI, C. et al. Remodeling of Sperm Chromatin Induced in Egg Extracts of Amphibians. International Journal of Developmental Biology. 1994, Vol. 38, pages 209-216.	1-16
A	DIMITROV, S. et al. Remodeling Somatic Nuclei in Xenopus Laevis Egg Extracts: Molecular Mechanisms for the Selective Release of Histones H1 and H1o from Chromatin and the Acquisition of Transcriptional Competence. EMBO Journal. 1996, Vol. 15, No. 21, pages 5897-5906.	1-16
A	MAXSON, R. et al. Differential Stimulation of Sea Urchin Early and Late H2B Histone Gene Expression by a Gastrula Nuclear Extract after Injection into Xenopus laevis Oocytes. Molecular and Cellular Biology. March 1998, Vol. 8, No. 3, pages 1236-1246.	1-16
A	WANGH, L. et al. Efficient Reactivation of Xenopus Erythrocyte Nuclei in Xenopus Egg Extracts. Journal of Cell Science. 1995, Vol. 108, pages 2187-2196.	1-16
A	US 5,480,772 A (WANGH) 02 January 1996, column 23, line to column 24, line 13.	1-16

☒ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

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"E" earlier application or patent published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

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Date of mailing of the international search report

16 January 2003 (16.01.2003)

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# INTERNATIONAL SEARCH REPORT

PCT/US02/26798

## C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,830,651 A (CAULEY et al.) 03 November 1998, column 9, lines 3-5.	17



## Letters to Nature

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## Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors

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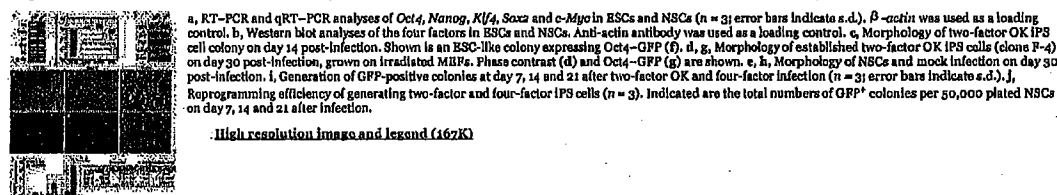
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Reprogramming of somatic cells is a valuable tool to understand the mechanisms of regaining pluripotency and further opens up the possibility of generating patient-specific pluripotent stem cells. Reprogramming of mouse and human somatic cells into pluripotent stem cells, designated as induced pluripotent stem (iPS) cells, has been possible with the expression of the transcription factor quartet Oct4 (also known as Pou5f1), Sox2, c-Myc and Klf4 (refs 1–11). Considering that ectopic expression of c-Myc causes tumorigenicity in offspring<sup>2</sup> and that retroviruses themselves can cause insertional mutagenesis, the generation of iPS cells with a minimal number of factors may hasten the clinical application of this approach. Here we show that adult mouse neural stem cells express higher endogenous levels of Sox2 and c-Myc than embryonic stem cells, and that exogenous Oct4 together with either Klf4 or c-Myc is sufficient to generate iPS cells from neural stem cells. These two-factor iPS cells are similar to embryonic stem cells at the molecular level, contribute to development of the germ line, and form chimaeras. We propose that, in inducing pluripotency, the number of reprogramming factors can be reduced when using somatic cells that endogenously express appropriate levels of complementing factors.

Mouse and human somatic cells can be reprogrammed into iPS cells by the expression of a defined set of factors (Oct4, Sox2, c-Myc and Klf4, as well as Nanog and human LIN28)<sup>1,2,3,4,5,6,7,8,9,10,11</sup>. It is possible to generate iPS cells from mouse and human fibroblasts in the absence of c-Myc retrovirus<sup>6,7</sup>, and therefore it was suggested that endogenous expression of c-Myc could have a role in the reprogramming process. Neural stem cells (NSCs) endogenously express Sox2, which may function in maintaining the stemness and multipotency of NSCs<sup>12,13</sup>, and Sox2 was suggested in maintaining cellular pluripotency by regulating the expression of Oct4 (ref. 14). NSCs were established from adult Oct4–GFP (OG2)/ROSA26 heterozygous transgenic mouse brains<sup>15,16,17</sup>, expressing green fluorescent protein (GFP) under the control of the Oct4 promoter (Oct4–GFP) and the *Escherichia coli lacZ* transgene from the constitutive ROSA26 locus (ROSA26 lacZ). The established NSCs had the capacity for self-renewal and multipotency (Supplementary Fig. 1).

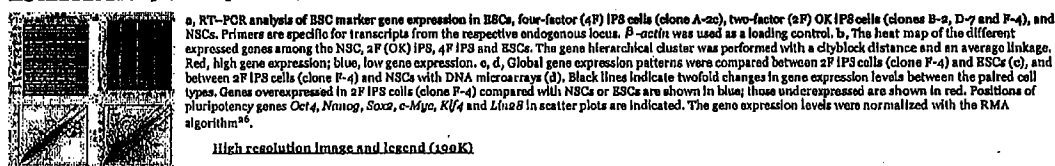
Compared to embryonic stem cells (ESCs), expression of Sox2 was approximately twofold higher in NSCs. c-Myc and Klf4 were also expressed at levels about tenfold higher and eightfold lower in NSCs than in ESCs, respectively (Fig. 1a). Western blot analyses showed that the relationship between protein and RNA levels in NSCs corresponded to that in ESCs for Oct4, Sox2 and Klf4; the c-Myc protein level was comparable in NSCs and ESCs (Fig. 1b).

**Figure 1: Generation of two-factor Oct4/Klf4 (OK) iPS cells from adult NSCs of OG2/ROSA26 transgenic mice.**



In this study, we attempted to reprogram NSCs into iPS cells by introducing different combinations of the four factors Oct4, Sox2, c-Myc and Klf4 (Supplementary Table 1) using the retroviral MX vector system. We assessed the ability of 15 different transcription factor combinations to induce Oct4–GFP-positive colony formation. Six combinations were able to induce the generation of iPS cells from NSCs, as judged by the formation of GFP<sup>+</sup> colonies and the establishment of iPS cell lines. We observed GFP<sup>+</sup> cells 4 days after transduction with the combination containing all four factors—that is, the control combination—and noted a gradual increase in the number of GFP<sup>+</sup> colonies during the first 2 weeks post-infection (Supplementary Fig. 2a). We established four-factor iPS cells from GFP<sup>+</sup> ESC-like colonies on day 14. These four-factor iPS cells stained positive for stage-specific embryonic antigen-1 (SSEA-1) and alkaline phosphatase (Supplementary Fig. 2b), showed messenger RNA expression patterns similar to those in ESCs (Fig. 2a, Supplementary Fig. 2c), and led to teratoma formation on injection into nude mice (Supplementary Fig. 2d). Our results demonstrate that NSCs can be reprogrammed into iPS cells by the four transcription factors: Oct4, Sox2, c-Myc and Klf4.

**Figure 2: Gene expression profile of iPS cells.**



Three different combinations of three factors were also capable of generating IPS cells from NSCs: *Oct4*, *Klf4* and *c-Myc* (OKM); *Oct4*, *Klf4* and *Sox2* (OKS); and *Oct4*, *c-Myc* and *Sox2* (OMS; **Supplementary Table 1**). We did not observe GFP<sup>+</sup> colonies for the three-factor combinations that did not include *Oct4*. GFP<sup>+</sup> colonies were observed 1 week after transduction with the OKM combination (without *Sox2*). However, GFP<sup>+</sup> colony formation was observed only after 14–21 days with the OKS combination (without *c-Myc*), and was even more delayed with the OMS combination (without *Klf4*; **Supplementary Table 1**). Nonetheless, these OKM, OKS and OMS three-factor IPS cells had similar gene expression profiles to ESCs (**Supplementary Fig. 3a**), and all types of three-factor IPS cells differentiated into all three germ layers (**Supplementary Fig. 3b**). Taken together, these results indicate that three-factor IPS cells could be generated in the absence of *Sox2*, *Klf4* or *c-Myc* retroviruses in NSCs, which endogenously express these three factors.

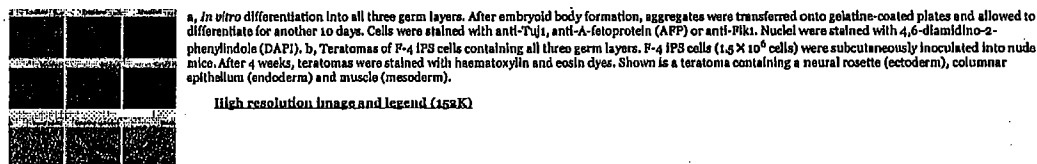
We next assessed the ability of two-factor combinations to induce the generation of IPS cells from NSCs. Only two combinations were successful in reprogramming NSCs. We first observed GFP<sup>+</sup> colonies in NSC cultures infected with *Oct4* and *Klf4* (OK) and 1–2 weeks later in those infected with *Oct4* and *c-Myc* (OM; **Supplementary Table 1**). The two-factor OM IPS cells showed an ESC-like gene expression pattern and contributed to the three germ layers in teratomas (**Supplementary Fig. 3a, b**). *Klf4* and *c-Myc* may exert similar functions. It is known that *Klf4* has a role in the inactivation of the *p53* (also known as *Trp53*) tumour-suppressor gene, which leads to cell immortalization; *Klf4* also works in conjunction with the RAS<sup>V12</sup> oncogenic signal transduction protein to stimulate cellular proliferation<sup>18,19</sup>. Similarly, the immortalizing gene product *c-Myc*, in conjunction with mutant RAS, exhibits an oncogenic effect<sup>20</sup>. It has been reported that *c-Myc* increases telomerase activity in NSCs, a mechanism possibly responsible for the immortalization of NSCs<sup>21</sup>. Because *c-Myc* increases tumorigenicity in chimera pups<sup>2</sup>, the recent studies demonstrating IPS cell generation without the *c-Myc* retrovirus<sup>6,7</sup> represent a significant finding. Importantly, our results of inducing IPS cells from NSCs with *Oct4* and *Klf4* and without *c-Myc* bring us even closer to the generation of IPS cells suitable for transplantation.

We compared two-factor OK IPS cells with four-factor IPS cells and ESCs. On day 14 post-infection, five GFP<sup>+</sup> colonies were dissociated and propagated under ESC culture conditions (**Fig. 1e, f**), yielding three (that is, 60%) two-factor OK IPS cell clones (B-2, D-7 and F-4) that were morphologically indistinguishable from ESCs (**Fig. 1d, g**). No colonies formed from NSCs infected with control virus (MX) (**Fig. 1e, h**). We estimated the reprogramming efficiencies from the number of *Oct4*-GFP<sup>+</sup> colonies and transduction rates with MX-GFP control virus on NSCs for the two-factor OK IPS and the four-factor IPS by time course (**Fig. 1i, j**). Thereby, we calculated a reprogramming efficiency of 3.6% for four-factor reprogramming of NSCs and 0.11% for the two-factor approach, which is comparable to reprogramming of fibroblasts with selection (<0.08%)<sup>1,2,3</sup> and without selection (0.5%)<sup>5</sup> (**Fig. 1j** and **Supplementary Table 2**). Transduction with all four factors had a positive impact on the timing and number of GFP<sup>+</sup> colonies. Integration of the viral transgenes was confirmed by genotyping by polymerase chain reaction (PCR; **Supplementary Fig. 4**). The viral transgenes of all four factors were detected in four-factor IPS cells, whereas two-factor OK IPS cells only contained the *Oct4* and *Klf4* transgenes.

Two-factor OK IPS cells stained positive for SSEA-1 and alkaline phosphatase (**Supplementary Fig. 5**), and exhibited expression patterns of ESC marker genes similar to those in four-factor IPS cells and ESCs (**Fig. 2a**). Quantitative real-time PCR (qRT-PCR) results demonstrated that expression of endogenous *Oct4*, *Sox2*, *c-Myc* and *Klf4* in two-factor OK IPS cells was comparable to that in ESCs (**Supplementary Fig. 6a**), and showed the silencing of the viral transcripts in two-factor OK IPS cells with a 1,000-fold reduction after 30 days (**Supplementary Fig. 6b**). In addition, in four-factor IPS cells, the endogenous expression levels were similar to those in ESCs, and the expression of the transgenes was completely silenced (**Supplementary Fig. 6c, d**). Global gene expression of two-factor IPS also clustered close to ESCs and four-factor IPS (**Fig. 2b** and **Supplementary Fig. 7**). Scatter plots of DNA microarray analyses demonstrated a higher similarity between two-factor IPS cells and ESCs than between two-factor IPS cells and NSCs (**Fig. 2c, d**). Thus, two-factor IPS cells (clone F-4) seemed to be very similar to mouse ESCs at the global transcription level.

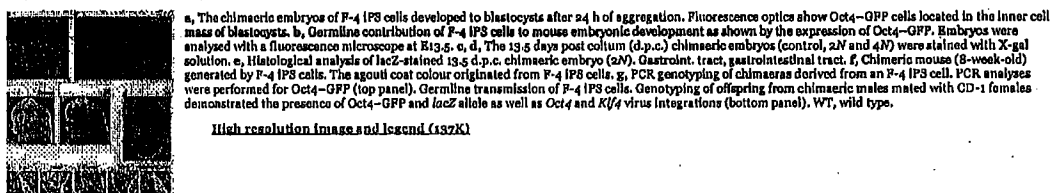
The differentiation ability of two-factor OK IPS cells was confirmed by *in vitro* differentiation into embryoid bodies. These cells expressed the ectoderm (*TuJ1*), endoderm (*A-fetoprotein*) and mesoderm (*Flk1*) markers (expressed by beating cells mimicking cardiomyocytes; **Fig. 3a** and **Supplementary Video 1**). Teratomas contained derivatives of all three germ layers (**Fig. 3b**) and expressed markers of the three germ layers (**Supplementary Fig. 8**). No teratoma had formed from donor cells (NSCs). These data demonstrate that two-factor OK IPS cells exhibit a pluripotent phenotype *in vitro* and *in vivo*.

**Figure 3: Two-factor *Oct4/Klf4* (OK) IPS cells (clone F-4) are pluripotent and differentiate *in vitro* and *in vivo*.**



To investigate their developmental potential, two-factor OK IPS cells were aggregated with 8-cell-stage embryos. IPS cells had contributed to the formation of the inner cell mass in developing blastocysts (**Fig. 4a**). After transferring aggregated blastocysts into pseudopregnant females, we obtained 16 live embryos on embryonic day (E)13.5, 2 of which showed germ cell contribution in the foetal gonads, judged from *Oct4*-GFP expression (**Fig. 4b**).  $\beta$ -galactosidase staining (visualizing the NSC donor cells that carry the *ROSA26 lacZ* gene) of embryonic tissue from whole embryos revealed that in the resulting chimaeras two-factor OK IPS cells contributed to the development of all three germ layers (**Fig. 4c, e**). The strictest test for developmental potency, tetraploid (4N) embryo aggregation ( $n = 122$ ), resulted in 2 dead (arrested) embryos at E13.5 (**Fig. 4d**). These data demonstrate that IPS cells can give rise to all of the tissues of a late-stage embryo. In diploid (2N) aggregation, PCR genotyping showed that 2 out of 13 chimaeras were positive for the *Oct4*-GFP allele of donor cells (**Fig. 4f, g**, top panel). To assess whether two-factor OK IPS cells can contribute to the germ line, chimaeras were mated with CD-1 females. Two out of 12 pups had a *Oct4*-GFP allele and 1 out of 12 mice had a *lacZ* allele, because the donor cells are derived from a heterogeneous mouse (*Oct4-GFP<sup>+/+</sup>ROSA26<sup>+/+</sup>*), and they also had the *Oct4* and *Klf4* transgenes (**Fig. 4g**, bottom panel). No tumour formation was observed from adult chimaeras and F<sub>1</sub> mice by the age of 21 weeks and 7 weeks, respectively. This finding indicates that two-factor OK IPS cells can contribute the full-term development of chimaera, resulting in a next generation (F<sub>1</sub>) of viable pups, and thus suggests that the IPS cells have a similar developmental property to that of ESCs.

**Figure 4: *In vivo* developmental potential of two-factor *Oct4/Klf4* (OK) IPS cells (clone F-4).**



In summary, our study demonstrates that Oct4 together with either Klf4 or c-Myc can reprogram NSCs into pluripotent stem cells. This is the first demonstration of an ectodermal cell type to be reprogrammed by defined factor overexpression. We propose that endogenous expression of Sox2 plus exogenous expression of two factors including Oct4 is sufficient to induce the generation of IPS cells from adult NSCs. However, it is not clear why exogenous expression of either Klf4 or c-Myc is still required for reprogramming, because these genes are endogenously expressed in NSCs.

Human NSCs might be available from patient biopsy<sup>22</sup>, and it will be particularly interesting to assess whether they can be reprogrammed with the currently described two-factor method. The clinical applicability of cellular reprogramming is incumbent on the optimization of approaches that obviate problems caused by retroviral insertional mutagenesis. Chromatin-modifying agents, such as trichostatin A and 5-Aza-2'-deoxycytidine, induce the transient expression of several pluripotency-associated genes, including Oct4 and Klf4 (ref. 23). This indicates that one or more reprogramming factor (s) could be replaced by such chemical compounds and, in this regard, the two-factor transcription factor reprogramming induction model holds great potential.

#### Methods Summary

NSCs were isolated from OG2/ROSA26 (Oct4-GFP) transgenic mice and transduced with 15 combinations of retroviral vectors expressing Oct4, Sox2, Klf4 and c-Myc as described<sup>1,24</sup>. Oct4-GFP-positive colonies were mechanically isolated and cultured on irradiated MEFs in ESC medium without any further selection. IPS cells were characterized by qRT-PCR and gene expression profiling by microarray. *In vitro* differentiation by embryoid body formation, the teratoma assay on injection into nude mice and chimera formation by aggregation with 8-cell-stage mouse embryos were used to measure the developmental potential of the IPS cells.

**Full methods** accompany this paper.

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#### Supplementary Information

**Supplementary information** accompanies this paper.

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**Author Contributions** J.B.K. (project design, generation and characterization of IPS cells and NSCs, and preparation of manuscript), H.Z. (project design, generation of IPS cells, and preparation of manuscript), G.W., L.G., V.S., K.K. and D.W.H. (characterization of IPS cells), M.J.A.-B., D.R., M.Z. (microarray and bioinformatics) and H.R.S. (project design, and preparation of manuscript).

### Author Information

The microarray data sets are available from the GEO (Gene Expression Omnibus) website under accession number [GSE10806](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE10806).

### Online Methods

#### Mice

The OG2 strain was crossed with the *ROSA26* transgenic strain<sup>16, 25</sup> over several generations to produce compound homozygous mice for the *neo/lacZ* and *Oct4-GFP* transgenes. To derive NSCs, homozygous OG2 × *ROSA26* male mice were crossed with ICR mouse strain females to produce heterozygous pups. Brain tissue was collected from 5-day-old OG2 × *ROSA26* heterozygous mice.

#### Generation of induced pluripotent stem cells

IPS cells and ESCs were grown on irradiated MEFs and in ESC medium (DMEM supplemented with 15% FBS, non-essential amino acids, L-glutamine, penicillin/streptomycin,  $\beta$ -mercaptoethanol, and 1,000 Units ml<sup>-1</sup> leukaemia inhibitory factor (LIF)). pMX-based retroviral vectors encoding the mouse complementary DNAs of *Oct4*, *Sox2*, *Klf4* and *c-Myc*<sup>1</sup> were separately cotransfected with packaging-defective helper plasmids into 293 cells using Eugene 6 transfection reagent (Roche). Forty-eight hours later, virus supernatants were collected as described previously<sup>24</sup>. NSCs derived from OG2/*ROSA26* transgenic mice were seeded at a density of  $5 \times 10^4$  cells per 6-well plate and incubated with virus-containing supernatants for the four factors (1:1:1:1) or for *Oct4* and *Klf4* (1:1) supplemented with 6  $\mu$ g ml<sup>-1</sup> protamine sulphate (Sigma) for 24 h. Transduction efficiencies were calculated with the pMX-GFP control virus. Cells were re-plated in fresh neural expansion medium. Two days after infection, the cells were further subcultured on irradiated MEFs in ESC medium containing LIF without any further selection. *Oct4-GFP*-positive colonies were mechanically isolated, and individual cells were dissociated and subsequently replated onto MEFs. The colonies were selected for expansion.

#### qRT-PCR analysis

Total RNA was extracted from cells using the MiniRNasey Kit (Qiagen GmbH; <http://www.qiagen.com>) according to the manufacturer's instructions. Complementary DNA synthesis was performed with the High Capacity cDNA Archive Kit (Applied Biosystems GmbH; <http://www.appliedbiosystems.com>) following the manufacturer's instructions with a down-scaled reaction volume of 20  $\mu$ l. Transcript levels were determined using the ABI PRISM Sequence Detection System 7700 (Applied Biosystems) and the ready-to-use 5'-nuclease Assays-on-Demand. For each real-time amplification, the template was equivalent to 5 ng of total RNA. Measurements were performed in triplicate; a reverse-transcription-negative blank of each sample and a no-template blank served as negative controls. Amplification curves and gene expression were normalized to the housekeeping gene *Hprt1*, used as an internal standard.

Oligonucleotides were designed by the Taqman Assay-on-Demand for the detection of the following genes (Taqman primer ID): *Oct4* (Mm00658129\_gH), *Sox2* (Mm00488369\_s1), *c-Myc* (Mm00487803\_m1), *Klf4* (Mm00516104\_m1)  $\beta$ -actin (Mm00607939\_s1) and *Hprt1* (Mm00446968\_m1). Oligonucleotides for the detection of *Nanog* and the viral sequences were custom-designed. Quantification was normalized on the endogenous *Hprt1* gene within the log-linear phase of the amplification curve obtained for each probe/primers set using the  $\Delta\Delta C_t$  method (ABI PRISM 7700 Sequence Detection System, user bulletin number 2).

#### Primer sequences for viral-specific qRT-PCR

pMXs-*Oct4* PF: 5'-TGGTACGGGAAATCACAAGTTTG, PR: 5'-GTCATAGTTC CTGTGGTGAAAGTTCA, probe: 5'-6FAM-CTTCACCATGCCCTCA-MGB. pMXs-*Sox2* PF: 5'-GTGTGGTGGTACGGGAAATCAG, PR: 5'-TTCAGCTCCGT CTCCATCATG, probe: 5'-6FAM-TGTACAAAAAAGCAGGCTTGT-MGB. pMXs-*Klf4* PF: 5'-GTGTGTGGTACGGGAAATCA, PR: 5'-CGCGAAGCTGG AGAA GGA, probe: 5'-6FAM-CTTCACCATGGCTGTGAG-MGB. pMXs-*cMyc* PF: 5'-TGGTACGGGAAATCACAAGTTTG, PR: 5'-GTCATAGTTC CTGTGGTGAAAGTTCA, probe: 5'-6FAM-CTTCACCATGCCCTCA-MGB. *Nanog* PF: 5'-AACCAGTGGTTGAATACTAGCAATG, PR: 5'-CTGCAATGGAT GCTG GGATACT, probe: 5'-6FAM-TTCAGAA GGGCTCAGCAC-MGB.

#### Microarray analysis

The microarray study was carried out using Mouse Genome 430 2.0 GeneChip arrays (Affymetrix) essentially as described previously<sup>23</sup>. In brief, total RNA was extracted from cells with the RNeasy kit including DNase digestion (Qiagen). Biotin-labelled cRNA was obtained from 3  $\mu$ g of total RNA with the GeneChip One-Cycle labelling kit (Affymetrix). Fifteen micrograms of cRNA were fragmented and hybridized to Affymetrix 430 2.0 GeneChip arrays at 45 °C for 16 h. DNA chips were washed, stained and scanned using an Affymetrix Fluidics device and a GC83000 scanner, and the images obtained were analysed using the GCOS software. The experiment was performed in triplicates for the ESCs and IPS cells and in duplicates for the NSCs. Normalization was calculated with the RMA algorithm<sup>26</sup> implemented in BioConductor.

#### In vitro differentiation of IPS cells

*Oct4-GFP* cells were harvested by fluorescence-activated cell sorting analysis and used for *in vitro* differentiation in embryoid bodies, which was performed with hanging drop in ESC medium without LIF. After 3 days, embryoid bodies were plated onto gelatine-coated 4-well dishes for another 10 days. The cells were stained with anti-TuJ1 antibody (1:100; Chemicon), anti-A-fetoprotein (AFP) antibody (1:100; R&D Systems), or anti-Flk1 antibody (1:100; R&D Systems).

#### Western blot analysis

Total cell lysates ( $2 \times 10^6$ ) prepared from the ESC and NSC were subjected to western blot analysis for expression of *Oct4* (Santa Cruz), *Sox2* (Santa Cruz), *Klf4* (Abcam) and *c-Myc* (Abcam).  $\beta$ -actin expression levels in all the samples were used as the loading control (Abcam).

#### SSEA-1 and alkaline phosphatase staining

SSEA-1 and alkaline phosphatase staining was performed with the ES Cell Characterization Kit (Chemicon) according to the manufacturer's

protocol.

#### Teratoma formation

iPS cells and NSCs ( $1.5 \times 10^6$  cells per mouse) were injected subcutaneously into the dorsal flank of nude mice. Four weeks after the injection, teratomas that had formed were fixed overnight (approximately 12 h) in 4% PFA and were embedded in paraffin. Sections were stained with haematoxylin and eosin dyes.

#### Chimaera formation

iPS cells were aggregated and cultured with denuded post-compacted 8-cell-stage mouse embryos. In brief, 2-cell-stage embryos were flushed from mice ((C57BL/6  $\times$  C3H) F<sub>1</sub> females  $\times$  CD1 males) at 1.5 d.p.c. and placed in M2 medium and cultured in KSOM medium with 0.1% BSA overnight to the 8-cell stage. Clumps of loosely connected iPS cells (10–20 cells) from short trypsin-treated day-2 cultures were selected and transferred into microdrops of KSOM medium with 10% FCS under mineral oil; each clump was placed in a depression in the microdrop. Meanwhile, batches of 30 to 40 embryos were briefly incubated with acidified Tyrode's solution until the zona pellucida had disintegrated. A single embryo was placed onto the clump. All aggregates were assembled in this manner, and were cultured at 37 °C in an atmosphere of 5% CO<sub>2</sub> in air. After 24 h of culture, most aggregates had formed blastocysts. A total of 64 aggregated blastocysts (2.5 d.p.c.) were transferred into the uterine horns of five pseudopregnant mice (CD-1 background).

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ORIGINAL ARTICLE

# Induction of stem cell-like plasticity in mononuclear cells derived from unmobilised adult human peripheral blood

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**Key words:** Haematopoietic, cardiomyocyte, neuronal progenitor/stem cells – CR3/43 monoclonal antibody – Embryoid body – Mononuclear cells – Pluripotency, transdifferentiation – Retrodifferentiation – Undifferentiated/embryonic stem cells

## SUMMARY

Undifferentiated pluripotent stem cells with flexible developmental potentials are not normally found in peripheral blood. However, such cells have recently been reported to reside in the bone marrow. Herein are reported methods of inducing pluripotency in cells derived from unmobilised adult human peripheral blood. In response to the inclusion of purified CR3/43 monoclonal antibody (mAb) to well-established culture conditions, mononuclear cells (MNC) obtained from a single blood donor are converted into pluripotent haematopoietic, neuronal and cardiomyogenic progenitor stem cells or undifferentiated stem cells. The haematopoietic stem cells are CD34<sup>+</sup>, clonogenic and have been shown to repopulate non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice. The neuronal precursors transcribe the primitive stem cell markers OCT-4 and nestin, and on maturation,

differentially stain positive for neuronal, glial or oligodendrocyte-specific antigens. The cardiomyogenic progenitor stem cells form large bodies of asynchronously beating cells and differentiate into mature cardiomyocytes which transcribe GATA-4. The undifferentiated stem cells do not express haematopoietic-associated markers, are negative for major histocompatibility complex (MHC) class I and II antigens, transcribe high levels of OCT-4 and form embryoid body (EB)-like structures. This induction of stem cell-like plasticity in MNC may have proceeded by a process of retrodifferentiation but, in any case, could have profound clinical and pharmacological implications. Finally, the flexibility and the speed by which a variety of stem cell classes can be generated *ex vivo* from donor blood could potentially transfer this novel process into a less invasive automated clinical procedure.

## Introduction

Somatic cell plasticity is an emerging field in stem cell biology. In this respect, a variety of more committed cells have been shown to re-switch their developmental potential in response to either nuclear transfer into an enucleated oocyte<sup>1</sup>, or to more remote micro-environmental cues or pressures, as is the case with stem

cells *in vivo*<sup>2-5</sup>. On the other hand, the phenomenon of cell transdifferentiation, during which more committed cells traverse the differentiation barrier and adopt a new specialisation fate *in vitro*, is well documented in the literature.

Transdifferentiation from one specialised fate to another has been demonstrated for many cell types: retinal pigmented epithelial cells into lens tissue or



retinal neuronal cells<sup>6,7</sup>; keratinocytes into mesenchymal myogenic-like cells<sup>8</sup>; dermal papilla cells into hair follicle epidermal cells<sup>9</sup>; squamous vaginal epithelial cells into cuboidal mucinous cells<sup>10</sup>; human pancreatic islet cells to pancreatic ductal cells<sup>11</sup>; fat storing cells into myofibroblasts<sup>12</sup>; chondrocytes into osteocytes<sup>13</sup>; oligodendrocyte precursors into multipotential CNS stem cells<sup>14</sup>; blood cells into brain<sup>4</sup> and vice versa<sup>5</sup> and pro-B cells into a variety of leukocyte subsets<sup>15</sup>. In most cases, the newly generated cells have been noted to redifferentiate into cells of the original lineage and stage.

While transdifferentiation is well documented, the underlying mechanisms remain poorly understood. The loss of differentiation markers, commonly termed dedifferentiation<sup>16</sup>, has been demonstrated in multinucleated heterokaryons<sup>17</sup>, a transfected cell line<sup>18</sup>, gut cells<sup>19</sup> and mammary epithelial cells<sup>1</sup>. However, explanations for dedifferentiation provided to date are ambiguous because they do not elucidate what is behind the loss of the differentiated state. Analyses of *de novo* gene activation in such cells suggest some sort of reprogramming.

Another mechanism proposed to explain the partial loss of the differentiated state of a cell is retrodifferentiation<sup>20-25</sup> which is defined in terms of what occurs during forward differentiation of a precursor cell. The principle underlying retrodifferentiation is the inversion of the differentiation programme to generate a cell at a progenitor or stem cell stage. In this form of retrograde development, a committed cell reverts to an earlier ontogenic stage. In contrast to transdifferentiation, evidence supporting retrodifferentiation is meagre, coming primarily from studies of cell lines such as myelomonocytic<sup>26</sup> and erythroid<sup>27</sup> leukaemias, regenerating liver cells<sup>28</sup> and neoplastic colon cells<sup>29</sup>. Such studies previously reported loss of differentiation markers, however, not to a stem cell stage.

Pluripotent stem cells with multi-developmental potentials are not found circulating in unmobilised adult human peripheral blood. These types of precursors have been reported recently to reside in the bone marrow<sup>30</sup>. The wide clinical indications where stem cell therapies are believed to benefit many patients with leukaemia, lymphoma, some solid tumours or degenerative diseases puts an added constraint on their current sources. Unmobilised adult peripheral blood contains insufficient quantities of stem cells to be used to treat such a wide spectrum of clinical indications. Herein, I report the *in vitro* production of a variety of stem cell classes derived from mononuclear cells (MNC) obtained from human adult peripheral blood in response to the addition of purified CR3/43 monoclonal antibody (mAb) to well-established culture conditions.

## Materials and methods

### Cell Culture

#### *Haematopoietic-Conductive Conditions (HCC)*

MNC were obtained from healthy human buffy coat samples (obtained from the National Blood Service, Brentwood, England) by density gradient centrifugation on Histopaque (Sigma) at a specific gravity of 1.077 g. After washing, MNC were resuspended at  $2 \times 10^6$  per ml in Dexter's long-term culture (LTC) medium<sup>31</sup> consisting of Iscove's Modified Dulbecco's Medium (IMDM) without phenol red (Invitrogen), 10% foetal calf serum (FCS), 10% horse serum (HS) (Sigma),  $10^{-7}$  M cortisol (StemCell Technologies), and 1% penicillin/streptomycin (Sigma) supplemented with 3.5 µg per ml of purified CR3/43 (DakoCytomation). This constituted the haematopoietic-conductive condition (HCC).

The CR3/43 clone was generated by DakoCytomation in the absence of azide and antibiotics. The CR3/43 mAb is raised against human monomorphic regions of the beta chain of the major histocompatibility complex (MHC) class II antigens DP, DQ and DR. This antibody binds to B cells, monocytes, antigen-presenting cells and activated T cells. Alternatively, the animal sera, cortisol and antibiotic components of the HCC culture medium can be replaced by citrated human autologous plasma (ACDA, Baxter Inc.) supplemented with 7 µg/ml CR3/43. In order to increase the yield of CD34<sup>+</sup> cells, human leukocytes enriched by dextran sedimentation or aphaeresed mononuclear fractions can be used instead of histopaque-separated MNC. Cells were plated in six-well plates (Fisher Scientific, USA) at 2 ml per well and incubated at 37°C and 5% CO<sub>2</sub> in air. The cells were fed the following day and, thereafter, every 4 days with Dexter's medium in the absence of CR3/43.

#### *Neuronal-Conductive Conditions (NCC)*

MNC were prepared and seeded as described above except that these were cultured in embryonic stem (ES) culture media<sup>31</sup> consisting of Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen), 20% FCS (Sigma), 1% L-glutamine (Sigma), 1% MEM non-essential amino acids (Invitrogen) and 0.2% of 0.1M β-2-mercaptoethanol (Sigma). Cells were fed the following day and thereafter every 4 days with ES medium in the absence of CR3/43.

#### *Cardiac Conductive Conditions (CCC)*

MNC were obtained as above and seeded as 20 µl 'hanging drops'<sup>33</sup> in ES<sup>32</sup> or LTC culture media<sup>31</sup>, as detailed above, containing 3.5 µg/ml purified CR3/43. Alternatively, MNC were subjected to HCC or NCC, minus cortisol and β-2-ME respectively, and seeded at

$2 \times 10^6$  per ml in six-well plates (Fisher Scientific USA) and chamber slides coated with 0.1% gelatin (StemCell Technologies). Beating areas in hanging drops<sup>33</sup> were observed using an inverted phase contrast microscope (Olympus CK-40) and imaged using a digital video camera.

### **Clonal Assays**

Cells were seeded in methocult GFH4434 according to manufacturer instructions (StemCell Technologies) containing recombinant human growth factors. Differentiation into haematopoietic cell colonies was assessed and colonies were inspected and scored with time using phase contrast microscopy (Olympus CK-40).

### **Purification of CD34 Cells by Positive Selection**

Twenty-four hours post-CR3/43 treated MNC under HCC were purified using the CD34 MultiSort Kit (Miltenyi Biotec) according to manufacturer's instructions. Briefly, prior to isolation, cells were subjected to Fc receptor blocking to prevent non-specific binding followed by direct labelling of cells with anti-human class II CD34-coated microbeads. The labelled cells were passed twice through an LD Midimacs separation column in the MACS separator and bound cells were gently flushed and collected for analysis.

### **Purification of Undifferentiated Cells by Negative Selection**

Twenty-four hours post-incubation, an aliquot of cells incubated under NCC were subjected to co-negative selection using anti-CD45 and anti-glycophorin A-coated magnetic microbeads and applied to the LD Midimacs column in the MACS separator all according to the manufacturer's instructions (Miltenyi Biotec). The unbound cells were retained, passed through a further magnetic separation step and collected as the CD45-negative/glycophorin A-negative population for analysis.

### **Confocal Microscopy**

In order to facilitate live imaging by confocal microscopy, cells cultured in HCC were plated in organ culture dishes whereby a cover slip formed an integral part of its base. Conjugated anti-human antibodies against CD19-fluorescein isothiocyanate (FITC) and class III CD34-Rphycoerythrin-Cy5 (RPE-Cy5) (both DakoCytomation) were added at the recommended dilutions directly to the reaction/cell mixture. Cells were imaged every 3 min for up to 12 h at room temperature. Imaging of colonies in methocult culture was performed once colonies had reached maturity by directly adding the following fluorescent conjugated anti-human antibodies to the culture media: glycophorin A-FITC; CD33-RPE-Cy5; CD61-FITC (all DakoCytomation).

### **Flow Cytometry**

Cultured cells harvested at specified time points were washed and resuspended in PBS containing 10% human AB serum (Sigma) or an Fc blocking reagent (Miltney Biotec) to block non-specific binding. Due to homotypic aggregation and adhesion induced in response to treatment with CR3/43, single-cell suspensions were obtained by continuous scraping, stirring and pipetting of the cultured cells. According to the manufacturer's instructions, cells were labelled for 15 min at 4°C with the following directly-labelled anti-human antibodies: class III CD34-PE, CD34-FITC and CD34-RPE-Cy5, CD38-PE, CD38-FITC, CD45-FITC, c-kit-PE, CD33-PE, CD61-FITC, glycophorin A-PE, CD19-PE and CD3-FITC (all DakoCytomation) and CD133-PE (Miltenyi Biotec). Autofluorescence and negative controls were determined throughout; isotype negative controls IgG1-FITC, IgG1-PE and IgG1-RPE-Cy5 (all DakoCytomation) were used. Cells were washed with cell wash (Becton Dickinson). Events ranging from 20 to 100 000 were acquired using FACScan (Becton Dickinson) and analysed using Cellquest software version 3.3.

### **Formation and Immunohistochemical Staining of Embryoid Body (EB)-Like Structures**

Twenty microliters containing 300 purified cells (as described above for negative selection) (i.e.  $1.5 \times 10^4$  cells per ml) were resuspended as 'hanging drop' cell cultures in ES medium<sup>33</sup>. EB-like structures were plucked from 'hanging drop' cultures and transferred to microscope slides pre-coated with poly-lysine. A coverslip was applied and the slides were incubated at -80°C for 15 min prior to fixing. In contrast, MNC cultured in NCC and CCC were directly fixed in chamber slides prior to blocking and staining. All samples were fixed for 15 min in 4% paraformaldehyde (Sigma). EB-like structures were stained using HRP-conjugated antibody for analysis by phase contrast microscopy. For HRP-conjugated staining, fixed embryoid body (EB)-like structures were incubated with peroxidase block (DakoCytomation) for 5 min and then further blocked and permeabilised using 0.15% Triton X-100/10% rabbit serum/PBS. Thereafter, EB-like structures were independently incubated with one of the following primary anti-human monoclonal antibodies: myocardial-specific actin; alpha-fetoprotein; cytokeratin-7 and -20; Desmin; S100 (all Dakocytomation) and pan-neurofilament (Sternberger Monoclonals) and then detected using a rabbit anti-mouse HRP (DakoCytomation), with all antibody dilutions recommended by the manufacturer. Nuclei were stained with haematoxylin (DakoCytomation). Faramount medium (DakoCytomation) was used for mounting and

the stained cells visualised by inverted phase contrast microscopy (Olympus CK-40). Imaging was subsequently performed using a digital camera attached to the microscope. Cystic formation by embryoid body was performed by staining suspension of intact cell clusters with DiI-C18 (kind gift from Dr Tim McCaffery). Following copious washing, embryoid bodies were analysed using fluorescence microscopy.

### Immunohistochemical Staining of Neuronal and Cardiomyogenic Development

MNC cultured in NCC and CCC were fixed as above, blocked using 0.15% Triton X-100/10% donkey serum/PBS and then differentially co-stained for neuronal and cardiac-specific markers respectively. For neuronal-specific staining, cells were co-stained with NF/GFAP, MAP-2/Tau or oligodendrocyte/CD45-FITC primary conjugate (DakoCytomation). In this case, primary anti-human monoclonal antibodies against pan-NF (Sternberger Monoclonals), MAP-2 (Sigma) and oligodendrocyte (Chemicon International) were detected using donkey anti-mouse RPE-Cy5 (Jackson Immunoresearch) whilst second-stage rabbit anti-human GFAP (DakoCytomation) and Tau (Chemicon International) were detected using donkey anti-rabbit FITC (Jackson Immunoresearch) and CD45 was directly stained with anti-human CD45-FITC conjugate (DakoCytomation).

MNC cultured in CCC were differentially co-stained for either cardiac-specific troponin I or myocardial-specific actin and human CD45. In this case, the primary anti-human monoclonal antibodies against troponin I (Fitzgerald Industries Inc.) and actin (Dakocytomation) were both detected using donkey anti-mouse RPE-Cy5 (Jackson Immunoresearch) whilst second-stage anti-human monoclonal antibody against CD45 was directly conjugated to FITC. In all cases, nuclei were stained using propidium iodide (PI) (Sigma). In addition, human NF, MAP-2 and GFAP were detected using TRITC-labelled rabbit anti-mouse conjugated antibody and nuclei stained with hoechst. Imaging was subsequently carried out by confocal microscopy.

### Reverse-Transcriptase PCR Analysis

Total RNA was isolated from cells at recorded time intervals using the RNazol reagent (Biogenesis) according to the manufacturer's instructions. The RNA was reverse transcribed using Moloney murine leukaemia virus reverse transcriptase (Promega). The reverse transcriptase products served as a template for independent PCR reactions using the thermostable Taq polymerase (Promega). For PCR analysis, OCT-4<sup>34</sup>, nestin<sup>35</sup>, CD34<sup>36</sup>, GATA4, hANP and cTnT<sup>37</sup> primers were used.

## Results

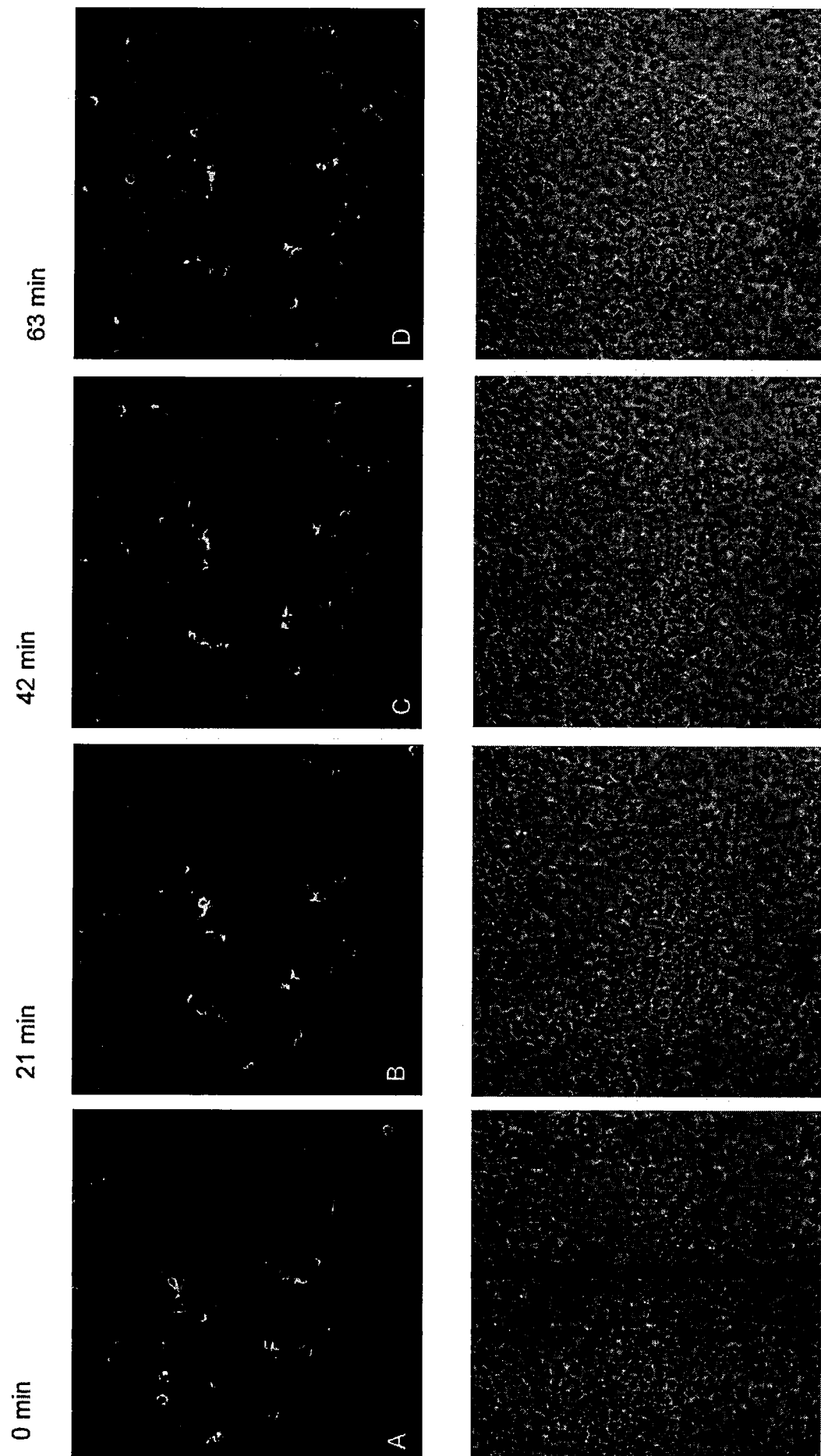
### Haematopoietic Analyses

#### *Live Image Analysis using Confocal Microscopy*

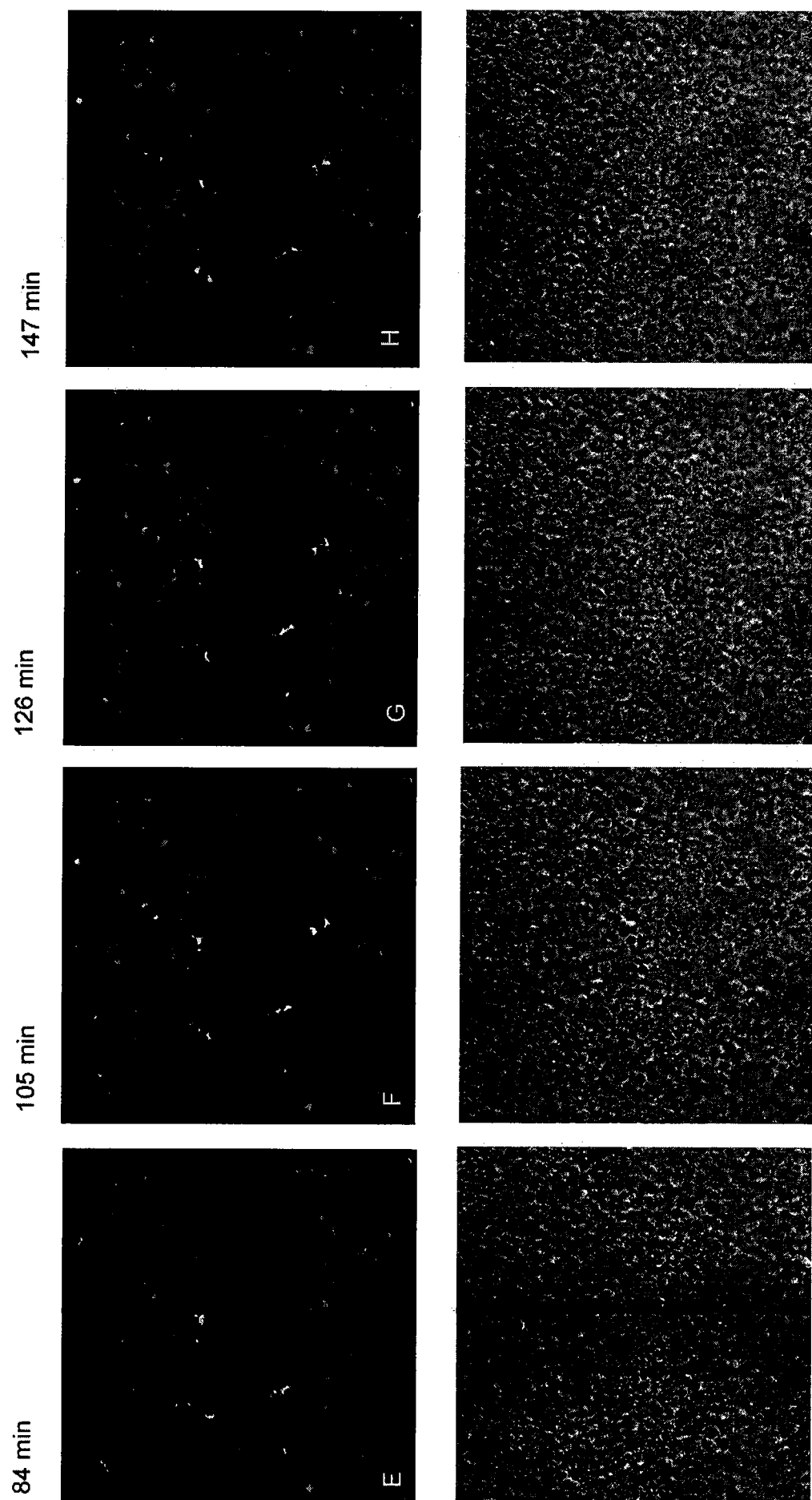
Treatment of MNC obtained from adult peripheral blood with purified CR3/43 mAb increases the relative number of CD34<sup>+</sup> cells cultured in Dexter's LTC medium<sup>31</sup>. The CR3/43 mAb is raised against human monomorphic regions of the beta chain of the major histocompatibility complex (MHC) class II antigens DP, DQ and DR. This antibody binds to B cells, monocytes, antigen-presenting cells and activated T cells. Under such HCC, live imaging of MNC using confocal microscopy (Figure 1A–L) reveals upregulation of the haematopoietic stem cell marker, CD34, concomitant with downregulation of the mature B lymphocyte marker CD19 (see supplementary data – real-time movie A<sup>38</sup>). These phenotypic changes occur within an hour of adding CR3/43 to the MNC cultured in Dexter's medium and are accompanied by cell motility in the culture dish.

#### Flow Cytometry Analysis

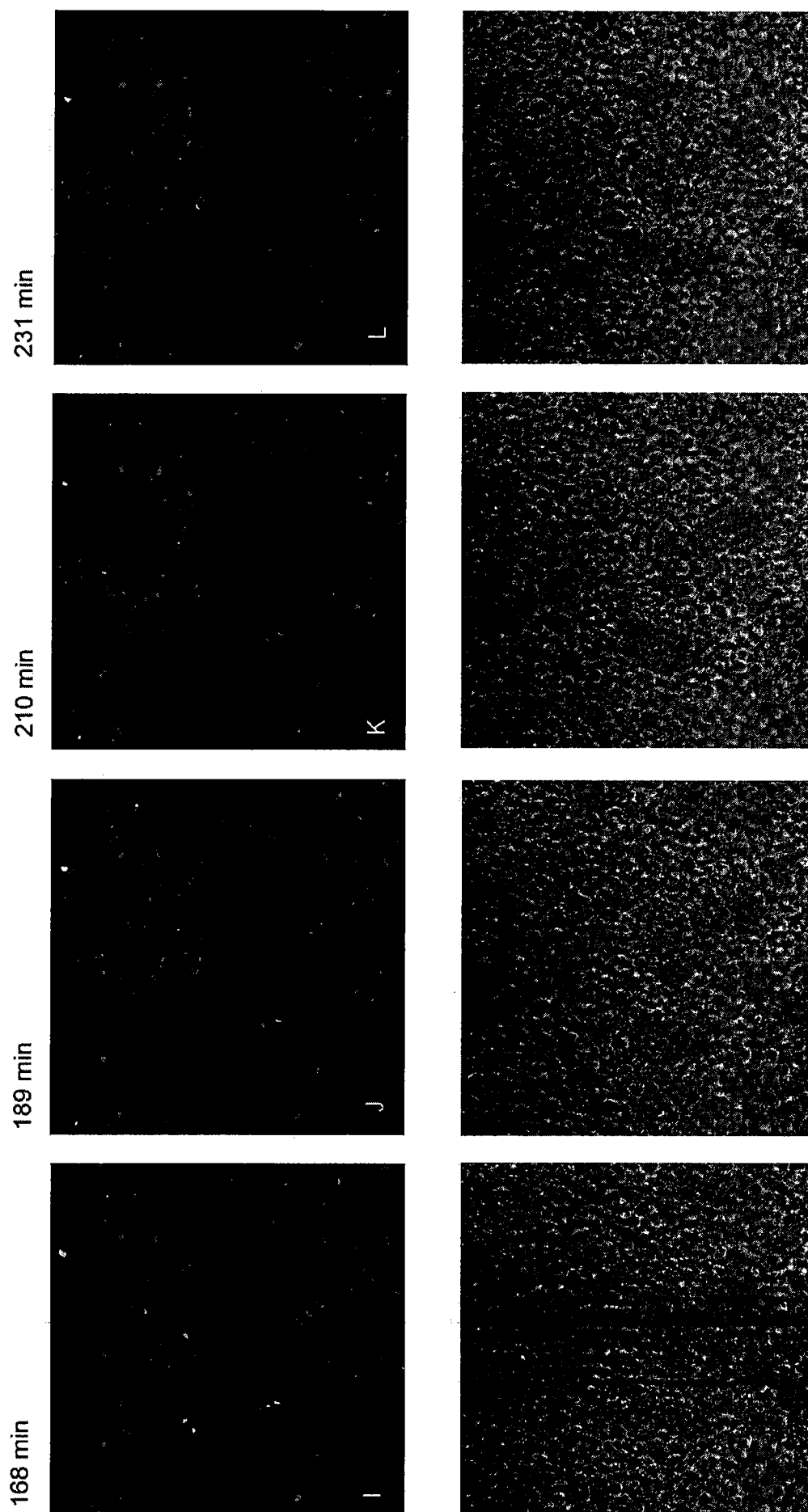
Immunophenotypic analysis of MNC under HCC before and after 2 h and 24 h treatment with CR3/43 mAb (Figure 2) shows significant increase in the relative number of cells expressing CD34 in response to treatment. The majority of CD34<sup>+</sup> cells are CD45 low and either positive or negative for CD38 antigen, typical of committed and more primitive haematopoietic progenitor cells<sup>39,40</sup>, respectively. The latter type has been shown to possess more long-term SCID repopulating potentials<sup>39</sup>. By 24 h in HCC, a significant proportion of CD34<sup>+</sup> cells co-express c-Kit or CD133 (Figure 2A). Furthermore, significant numbers of CD34<sup>+</sup> cells were purified from MNC cultured for 24 h in HCC, using well-established haematopoietic progenitor purification procedures<sup>41</sup> such as magnetic beads labelled with anti-human CD34 (Figure 2B). The purified CD34<sup>+</sup> cells exhibited, as for conventional haematopoietic stem cells, CD45 at low levels with or without CD133. The CD34<sup>+</sup> CD133<sup>+</sup> cells were more CD34 bright<sup>42</sup> than those cells that were CD34<sup>+</sup>CD133<sup>-</sup>. A significant proportion of CD34<sup>+</sup> cells were either CD38 positive or negative. The latter immunophenotype have been reported to possess more long-term SCID repopulating potential<sup>39</sup>. Moreover, significant levels of CD34 transcripts<sup>36</sup> were amplified from the purified CD34<sup>+</sup> cells and unfractionated MNC cultured for 24 h in HCC when compared to MNC cultured in LTC alone (Figure 2C).



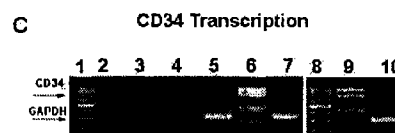
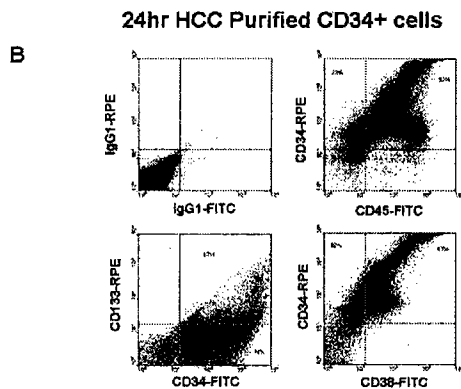
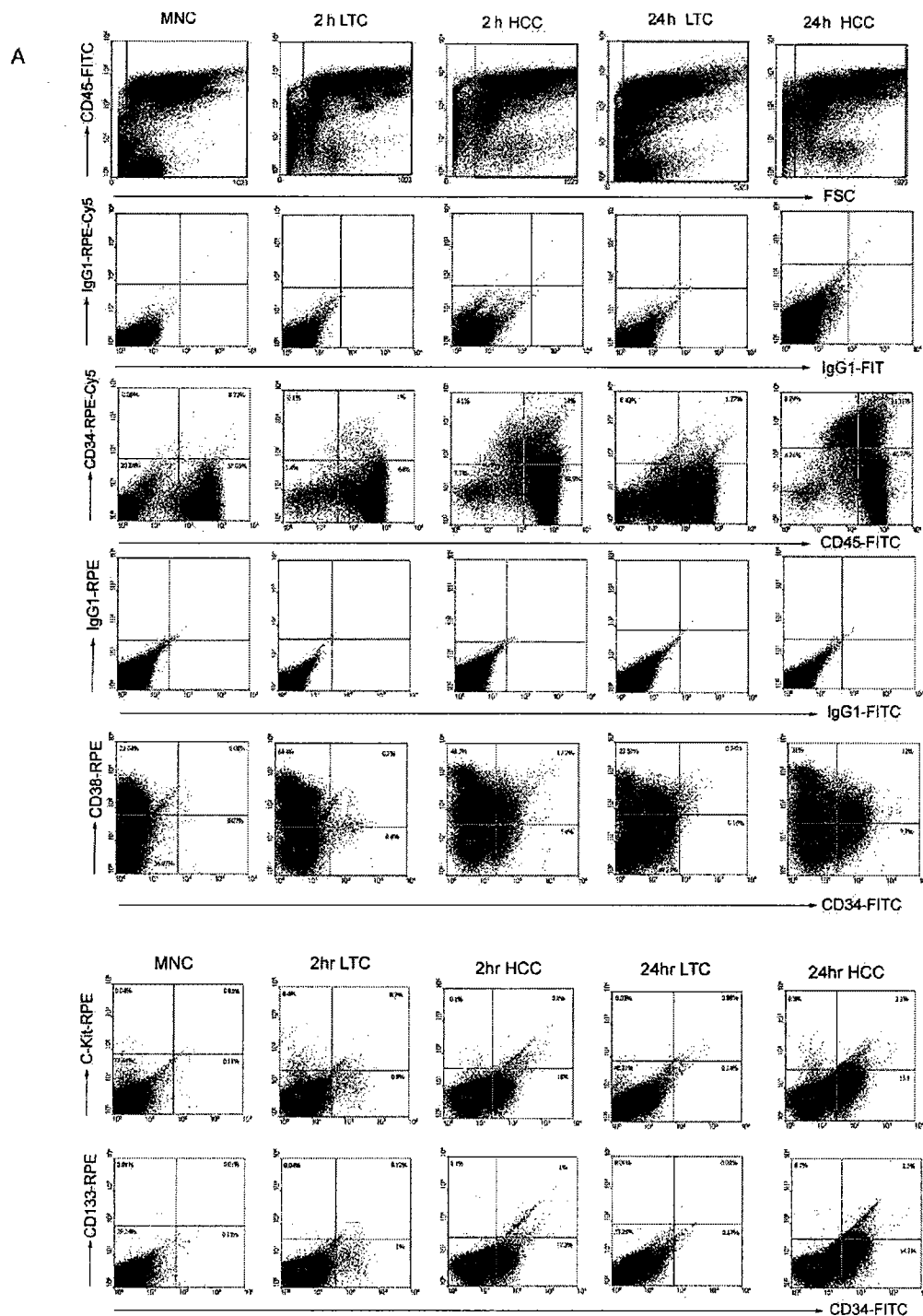
*Figure 1. (Continued on pages 360 and 361)*



*Figure 1. (Continued)*



**Figure 1.** Confocal microscopy showing the upregulation of CD34 concomitant with the down regulation of CD19 by MNC cultured in HCC. Cells were live imaged every 3 min for up to 12 h. Anti-human CD19-FITC (green) and CD34-RPE-cy5 (red) antibodies were added to the cell mixtures directly in order to visualise changes in cell receptor expression. Frames (A-E) are selected at 21-min intervals. Frame (A) and (B-L) are MNC before and after addition of CR3/43 mab to LTC respectively. Lower panels show the corresponding phase contrast image of the upper panels at the same time points. In addition, the superimposed real-time movie of the above images shows the movement of cells during up-regulation of CD34 (refer to Real-time movie A'\*)



Following cryopreservation of the 24 h MNC cultured in HCC and subsequent purification of CD34<sup>+</sup> cells, the yield of viable CD34<sup>+</sup> cells per one unit of buffy coat (500ml of donated blood) as measured by flow cytometry, and cell viability assessed by trypan blue dye exclusion assay, is approximately 100–150 × 10<sup>6</sup> cells and dependent on MNC input. For example, higher yields of MNC are obtained via aphaeresis or dextran sedimentation than when fractionated on a density gradient. Throughout culturing of MNC in HCC, cells remain viable and there is an approximate 1.6-fold increase in their absolute numbers 24 h later. On the other hand, 24 h culturing of MNC in LTC (not supplemented with CR3/43 mAb) gives rise to an increase (about 20%) in the number of erythrocytes and dead cells (Figure 2). Viability assessment of MNC cultured in HCC or LTC at 2 h and 24 h from culturing following cryopreservation, showed 30% cell death in cells cultured in LTC compared to 5% in the HCC group {data not shown}.

### Colony Formation

By 2 h in HCC, MNC were rendered clonogenic when single-cell suspensions were seeded in methocult-containing growth factors. They produced a variety of haematopoietic colonies such as colony-forming unit-granulocyte, erythroid, monocyte, macrophage, megakaryocyte (CFU-GEMM), colony-forming unit-granulocyte, monocyte, macrophage (CFU-GM), colony-forming unit-monocyte, macrophage (CFU-M), blast-forming unit-erythroid (BFU-E) and colony-forming unit-erythroid (CFU-E) (Figure 3). On the other hand, seeding MNC, cultured for 24 h in HCC, at the same cell density in methocult medium (basic or growth factor-containing) supplemented with 300 µl of 24 h HCC supernatant (to basic or growth factor-containing medium) resulted in the formation of much larger and wider variety of colonies, including megakaryocytes, indicating the secretion of cytokines by cells cultured in HCC (Table 1). On the other hand, the clonogenic efficiencies of purified CD34<sup>+</sup> cells obtained from MNC cultured for 24 h in HCC is 1 in 2.5 when compared to a value of 1 in 10<sup>5</sup> MNC cultured for 24 h in LTC {data not shown}. Direct immunostaining of these high proliferative potential<sup>43</sup> (HPP-CFC) haematopoietic colonies in methocult cultures with anti-human antibodies specific for myeloid and erythroid

antigens using confocal microscopy showed differential expression of glycophorin A without CD33, CD33 without CD61 and CD33 with CD61 (Figure 3R–T), a staining pattern typical of erythrocytes, monocytes/granulocytes and megakaryocytes, respectively. More significantly, ploidy or cell cycle analysis of fully differentiated haematopoietic colonies grown in methocult show normal DNA content {data not shown}. Furthermore, within 24 h, MNC cultured in HCC gave rise to cobblestone areas and stromal-like cells (Figure 3K). In addition, cryopreserved 2 h and 24 h MNC in HCC were clonogenic when compared to the same MNC population cultured in LTC. Interestingly, the time at which MNC became capable of repopulating non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice<sup>44</sup> at a relatively higher level and frequency was 3 h following the addition of purified CR3/43 mAb to MNC cultured in Dexter's medium {manuscript in preparation}.

### Purification and Characterisation of Undifferentiated Cells

Apart from red blood cells, leukocytes and extremely low levels of haematopoietic precursors, mature healthy blood is not known to contain undifferentiated cells or neuronal precursors. Treatment of MNC cultured in ES culture media<sup>32</sup> with 3.5 µg/ml purified CR3/43 mAb resulted, by 24 h, in an increase in the number of undifferentiated cells that were both CD45 and CD34 negative (Figure 4A). These cells were purified by negative selection on a Midi Macs column using anti-human CD45

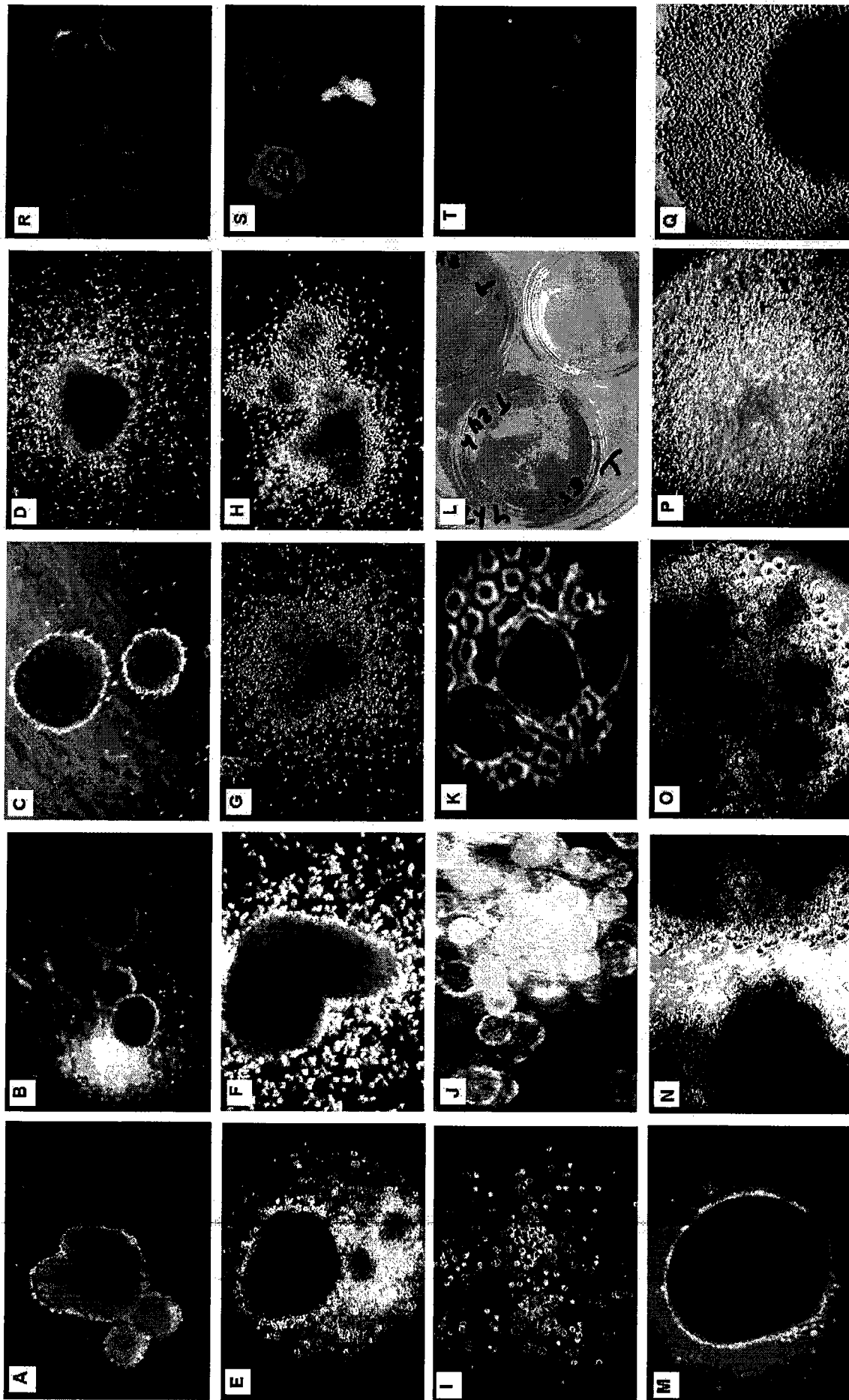
**Table 1.** Percentage of clonogenic progenitors within MNC cultured in HCC after 24 h (324 ± 37.9 colonies derived per 1 × 10<sup>4</sup> nucleated cells)

CFU-GEMM (%)	CFU-GM (%)	CFU-M (%)	BFU-E (%)	CFU-Meg (%)
47.9 ± 5.8	19.8 ± 3.9	5.4 ± 2.2	9.7 ± 2.0	16.0 ± 3.1

CFU-GEMM indicates colony-forming unit (CFU)-granulocytic, erythroid, monocyte, macrophage, megakaryocytic; CFU-GM, CFU-granulocytic, monocyte, macrophage; CFU-M, CFU-monocyte, macrophage; BFU-E burst-forming unit-erythroid and CFU-Meg, CFU-megakaryocytic. The percentage of each colony type is calculated as the mean ± standard error of mean (SEM) (n = 5). The number of phenotypically distinct progenitors were identified and measured from the use of plating conditions according to standard protocols

**Figure 2 (opposite).** Increase in the number of CD34<sup>+</sup> cells in response to MNC cultured in HCC (A). Cells were stained as indicated with a panel of conjugated monoclonal antibodies against human CD34, CD45 and CD38, c-kit and CD133 antigens before and after (2 h and 24 h) culturing MNC in HCC (Dexter's medium with CR3/43 mAb) and LTC (Dexter's medium alone). Cells analysed by FACScan were gated according to FSC and CD45 and in conjunction with the relevant isotype negative control. Purified CD34<sup>+</sup> cells from 24-h MNC cultured in HCC using the CD34 MultiSort Kit (B). Cells were stained with a panel of conjugated monoclonal antibodies against human CD34 and CD45, CD34 and CD38 and CD34 and CD133. (C) RT-PCR showing CD34 transcription by purified and unfractionated 24 h MNC cultured in HCC. Lanes 1 and 8, 1 KB ladder; lanes 2 and 3, GAPDH and CD34 gene transcript negative controls, respectively; lanes 4 and 5 are CD34 and GAPDH gene transcripts, respectively, in 24 h MNC/LTC; lanes 6 and 7 are CD34 and GAPDH gene transcripts, respectively, in purified CD34<sup>+</sup> cells from 24 h MNC/HCC; lanes 9 and 10 are CD34 and GAPDH gene transcripts, respectively, in unpurified 24 h MNC/HCC





**Figure 3.** Different types of clonogenic cells are produced in response to treatment of MNC in (A-K) HCC and (L-Q) NCC. Panels: (A-C) CFU-GEMM; (G, H) CFU-GM; (I) CFU-M; (J) CFU-Meg; (K) cobble stone areas and stromal like cells. In contrast, MNC cultured in NCC gives rise to colonies that resemble (M) embryoid bodies or (N, O) large erythroid or (P, Q) myeloid-containing colonies. Such large colonies are (L) visible to the naked eye. In addition, differential co-staining of colonies produced by MNC cultured in HCC demonstrates (R) glycophorin A-positive (green) and CD33<sup>+</sup> (red) (S) CD33<sup>+</sup> (red) and CD61<sup>+</sup> (green) co-positive and (T) CD33<sup>+</sup> (red) and CD61<sup>+</sup> (green) haematopoietic lineages typical of erythroid, megakaryocytic and granulocytic/monocytic cell lineages, respectively

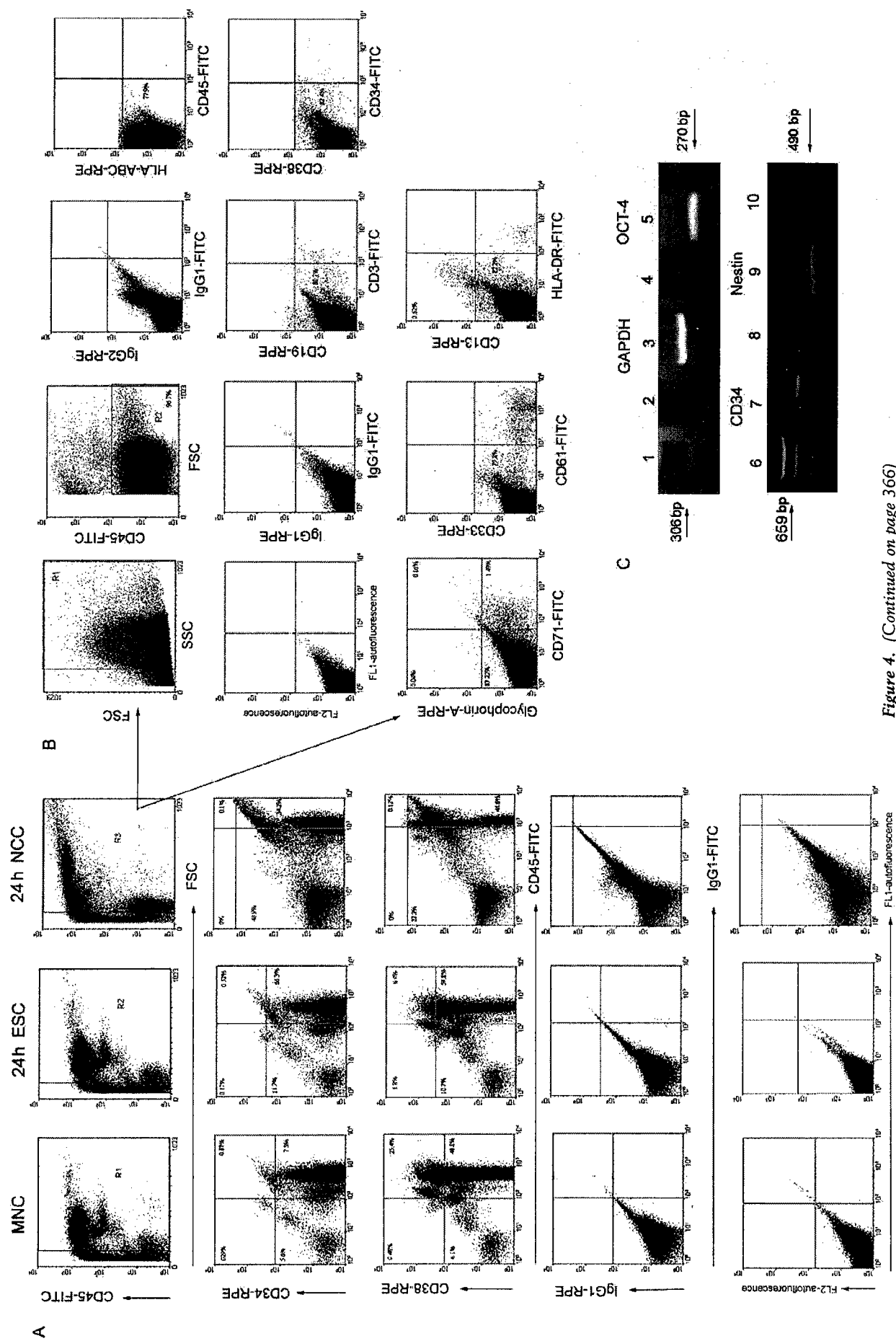
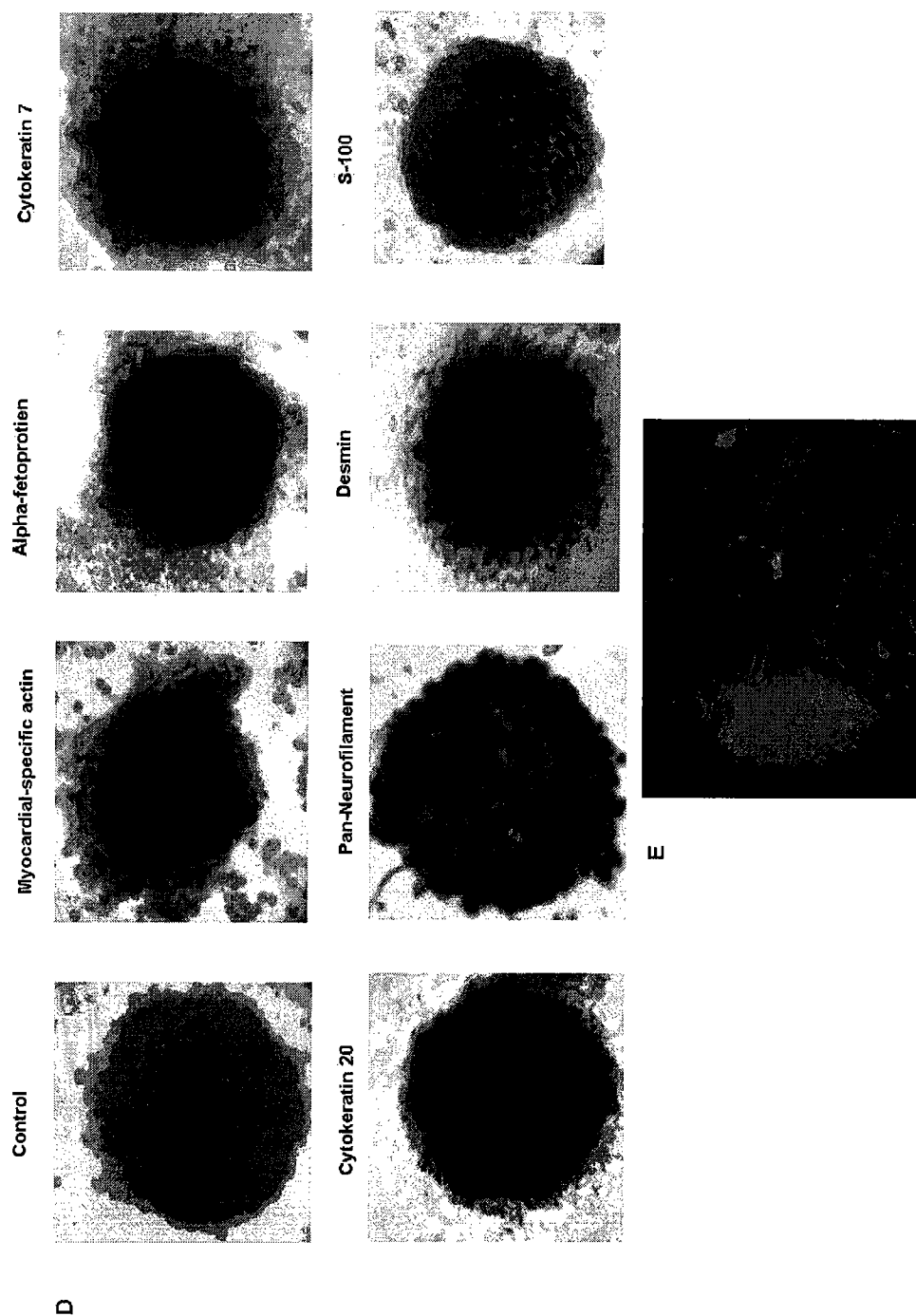


Figure 4. (Continued on page 366)



**Figure 4.** Increase in the numbers of undifferentiated CD45/lineage negative cells in response to MNC cultured in ES medium and supplemented with CR3/43 mab. For (A, B) FACSscan analysis, 24-h MNC cultured in ES medium with or without CR3/43 mab were harvested and stained as indicated with a panel of conjugated monoclonal antibodies against human (A) CD34, CD45 and CD38 antigens. (B, C) Characterisation of the purified undifferentiated CD45/lineage-negative cells by flow cytometry and RT-PCR, respectively. In (B) flow cytometry shows the purified cells are HLA-ABC-CD45-CD19-CD3-CD34-CD38-CD71-glycophorinA-CD33-CD61-CD13-HLA-DR negative. In (C) RT-PCR shows that the purified cells transcribe high levels of (lanes 3 and 5, respectively) GAPDH and OCT-4 and to a lesser extent (lanes 7 and 9 respectively) CD34 and nestin. Lanes 1 and 6 are 1 KB ladder, lanes 2, 4, 8 and 10 are negative controls for GAPDH, OCT-4 CD34 and nestin, respectively. (D) Immunohistochemical analysis of EB-like structures formed 48 h following culturing of the purified cells (characterised in B and C) as 'hanging drops' (resuspended as 300 cells per 20  $\mu$ l drop) show that they are positive for myocardial-specific actin, alpha-fetoprotein, cytokeratin 7 and 20, pan-NF, desmin and S-100 (brown stain) when compared to negative control EB-like structures stained with HRP conjugated secondary antibody alone. Nuclei were stained blue with haematoxylin. The stained cells were visualised by inverted phase contrast microscopy (Olympus CK-40). Imaging was subsequently performed using a digital camera attached to the microscope. (E) DiI-C18 labelled cystic embryoid body

and glycophorin A-coated magnetic beads (Miltenyi Biotec), in order to deplete leukocytes and red blood cells, respectively. The unbound cells were analysed by flow cytometry using a panel of anti-human monoclonal antibody conjugates. The purified cells were negative for haematopoietic-associated markers that are typically expressed by the erythroid, lymphoid and myeloid cell lineages and include MHC class I and II antigens (Figure 4B). In addition, such purified cells transcribed high levels of the embryonic stem cell marker, OCT-4, and, to a lesser extent, nestin and CD34 (Figure 4C). Following purification, 95% of these cells were viable and, moreover, one unit of buffy coat can yield up to  $90\text{--}130 \times 10^6$  of such undifferentiated cells.

Upon culturing as 'hanging drops'<sup>33</sup>, the purified cells formed cystic EB-like structures by 48 h that expressed ectodermal, endodermal and mesodermal antigens including alpha-fetoprotein (Figure 4D–E). Taken together, these analyses demonstrated the production of undifferentiated cells 24 h following the addition of CR3/43 mAb to MNC cultured in ES medium. Similarly, 24 h of culturing MNC in ES medium containing CR3/43 grown either on chamber slides (StemCell Technologies) or six-well plates resulted in the formation of EB-like structures that are loosely attached to the substratum and stain positive for ectodermal, mesodermal and endodermal antigens<sup>45,46</sup>. In addition, single-cell suspensions obtained from 24 h MNC cultured in ES medium containing CR3/43 mAb seeded in methocult medium (containing recombinant growth factors and incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> and 20% O<sub>2</sub>) produced uniform colonies akin to embryoid bodies (Figure 3M). In contrast the same cells, seeded in the same methocult at 37°C containing 5% CO<sub>2</sub> and 5% O<sub>2</sub>, formed larger colonies visible to the naked eye (Figure 3L) consisting of myelocytes and erythrocytes or myelocytes only (Figure 3, N–O and P–Q, respectively).

## Neuropoietic Analyses

Notably, continued culturing of MNC in ES medium initially containing CR3/43 mAb resulted in the eventual conversion of MNC into spherical bodies and neuronal-like cells (Figure 5A). By 24 h, MNC in NCC, transcribed OCT-4 and nestin (Figure 5B)<sup>34,35</sup> and on maturation stained positive for MAP-2, glial fibrillary acidic protein (GFAP) and neurofilament (NF) 200 and 70 by weeks 1 and 2 (Figure 5C). This process was always accompanied by downregulation of nestin and OCT 4.

Dual immunohistochemical staining of MNC in NCC performed at defined time points using antibodies to MAP-2 and Tau, NF and GFAP and oligodendrocyte and CD45, and analysed by confocal microscopy, showed differential expression of neuronal, glial and oligodendrocytic antigens<sup>47</sup>. For example, by 48 h, cellular

spheres formed that co-stained positive for MAP-2 and Tau, indicative of neurospheres containing immature neurons. One and 2 weeks later, the spheres differentiated into mature neurons, with MAP-2 being confined to cell bodies and Tau to the axons (Figure 5D). On the other hand, spheres that were analysed for NF and GFAP showed the presence of neuronal and glial precursors. However, 1 and 2 weeks later, such spheres differentiated into neurons expressing NF alone without GFAP, or astrocytic-like cells expressing GFAP without NF, the latter typical of glial cells (Figure 5E). In contrast, co-staining for the pan-leukocyte marker, CD45, and oligodendrocyte, indicated the absence of CD45 expression at all time points shown, while oligodendrocyte staining was confined to the periphery of the spheres and persisted in 1- and 2-week-old cultures (Figure 5F). Moreover, time course analysis of neurotransmitters released by these neurons in response to depolarisation revealed that, as they matured, the neurons produced increasing amounts of glutamate, GABA, tyrosine, dopamine and serotonin and uptake of taurine {unpublished data}.

## Cardiopoietic Analyses

The cardiomyogenic-conductive condition (CCC) involves culturing of MNC either as 'hanging drops'<sup>33</sup> or in HCC or NCC in six-well plates or chamber slides, coated with 0.1% gelatin. In 'hanging drops', MNC cultured in CCC formed compact embryoid-like bodies that started to beat in an asynchronous manner within 24 h from the initiation of such cultures. These large masses of cell aggregates were surrounded by novel single cells that aligned themselves along parallel axes while undergoing synchronous cyclical beating rhythms consisting of torsional contraction, elongation and rotation (see supplementary data – Real-time movie B<sup>48</sup>). These novel cells were found in the meniscus at the centre of the hanging drops. Interestingly, the relative number of contracting cells or embryoid-like structures in the 'hanging drops' increased significantly when cortisol and mercaptoethanol were removed from HCC or NCC, respectively. Moreover, azide was noted to be a powerful inhibitor of the beating cells. Within 1 week, MNC in CCC grown on gelatinised six-well plates or chamber slides transcribed the cardiac transcription factor GATA-4, human atrial natriuretic peptide (hANP) (Figure 6B) and cTnT<sup>37</sup>. Dual immuno-histochemical analysis of 1-week-old CCC-cultured MNC revealed the expression of myocardial-specific actin and cardiac-specific troponin I without CD45 expression (Figure 6A). More interestingly, 3-day and 7-day-old MNC cultured in CCC were able to differentiate into fully mature cardiomyocytes when infused into the myocardium of non-irradiated non-infarcted *Rnu/Rnu* nude rats<sup>49</sup> {unpublished data}.

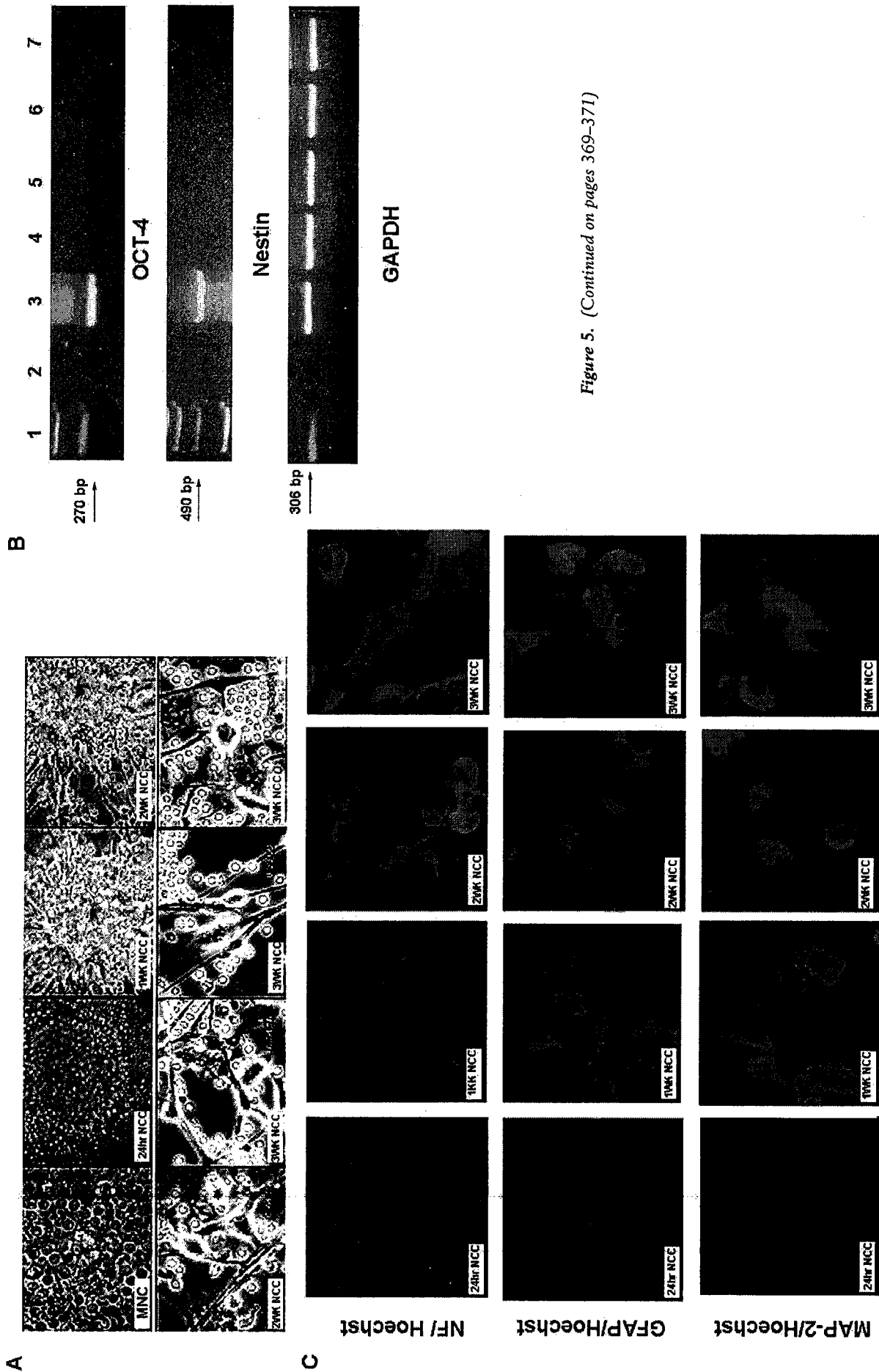


Figure 5. (Continued on pages 369-371)

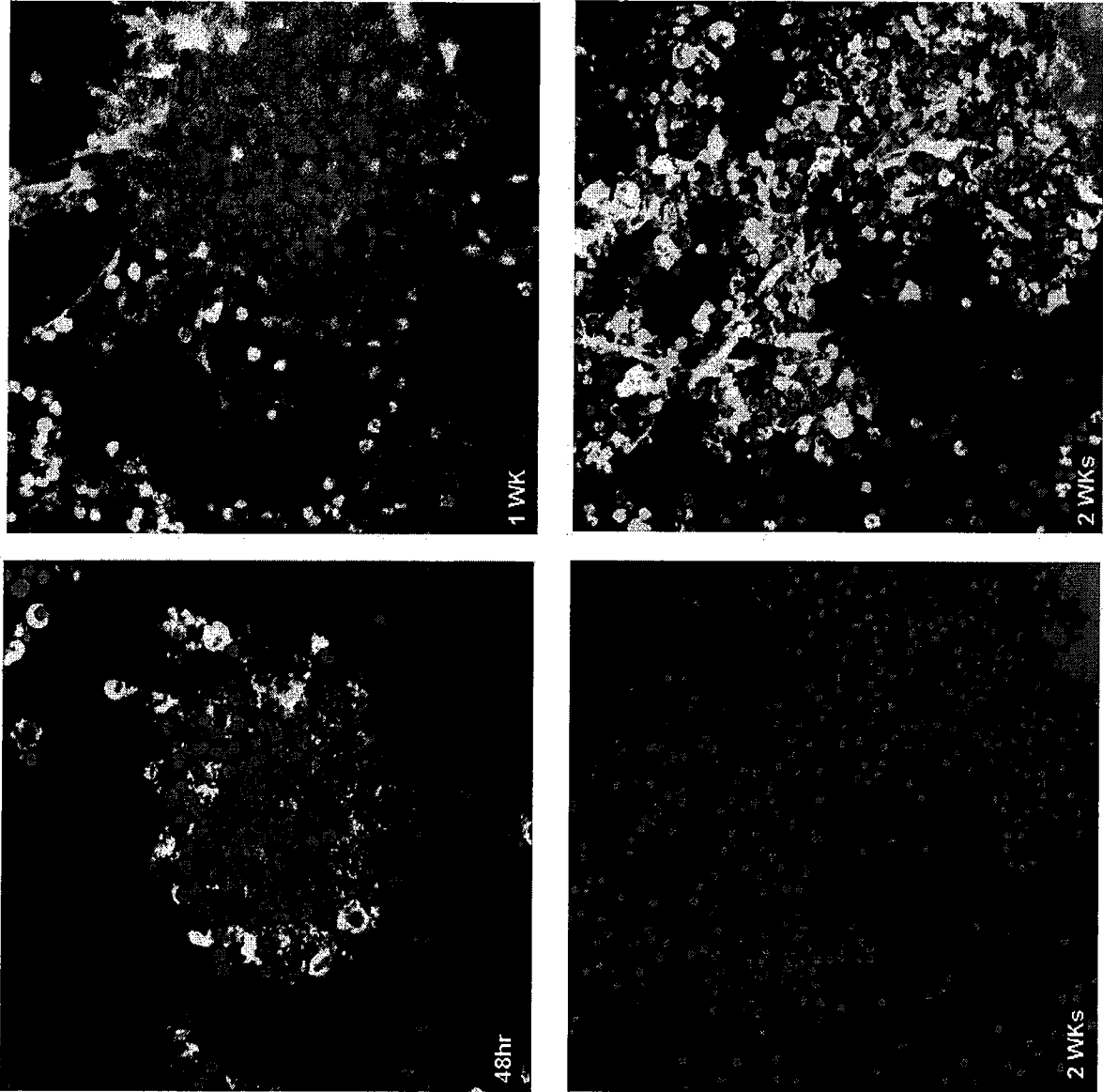


Figure 5. (Continued)

MAP-2 and Tau

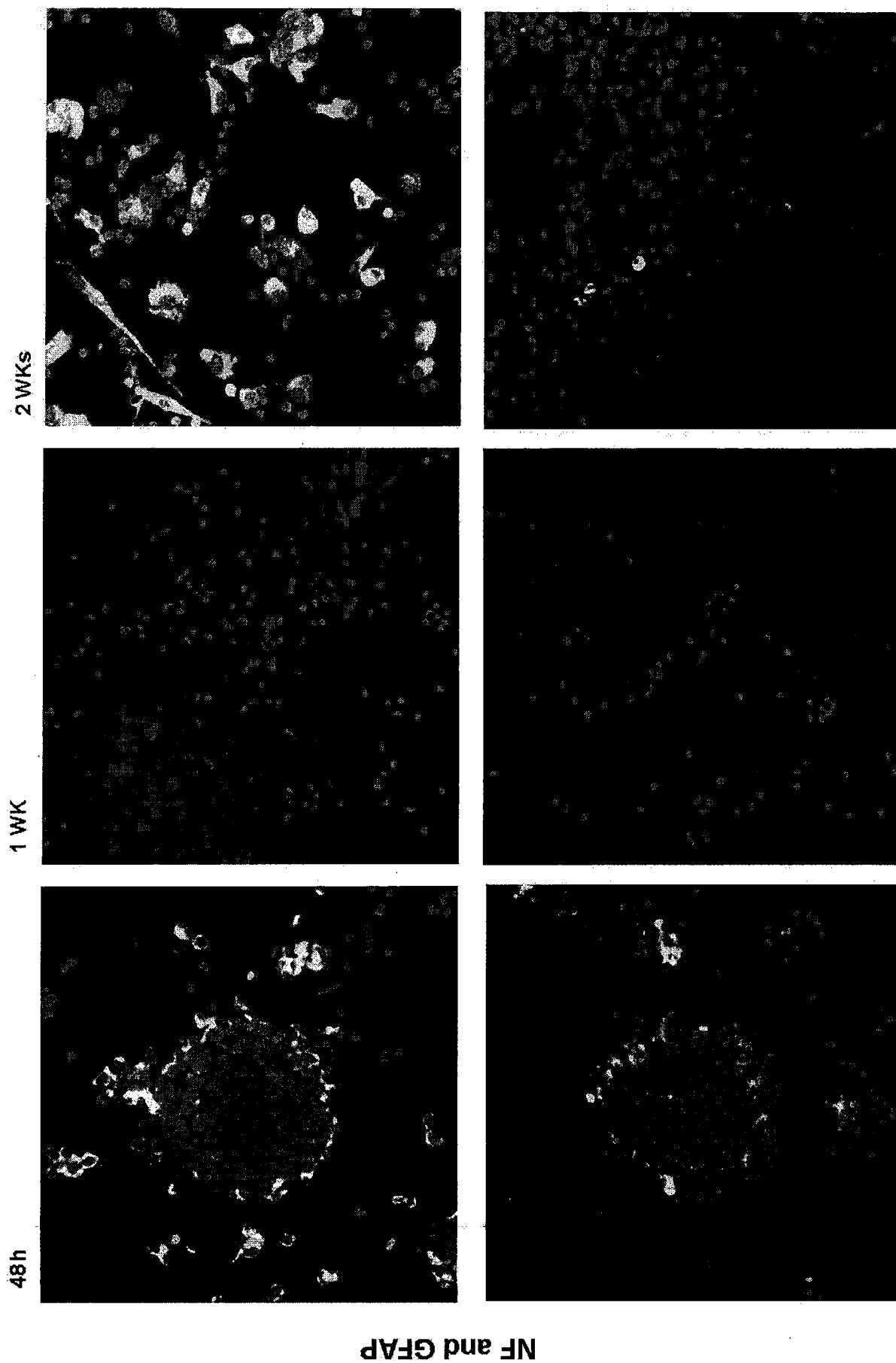
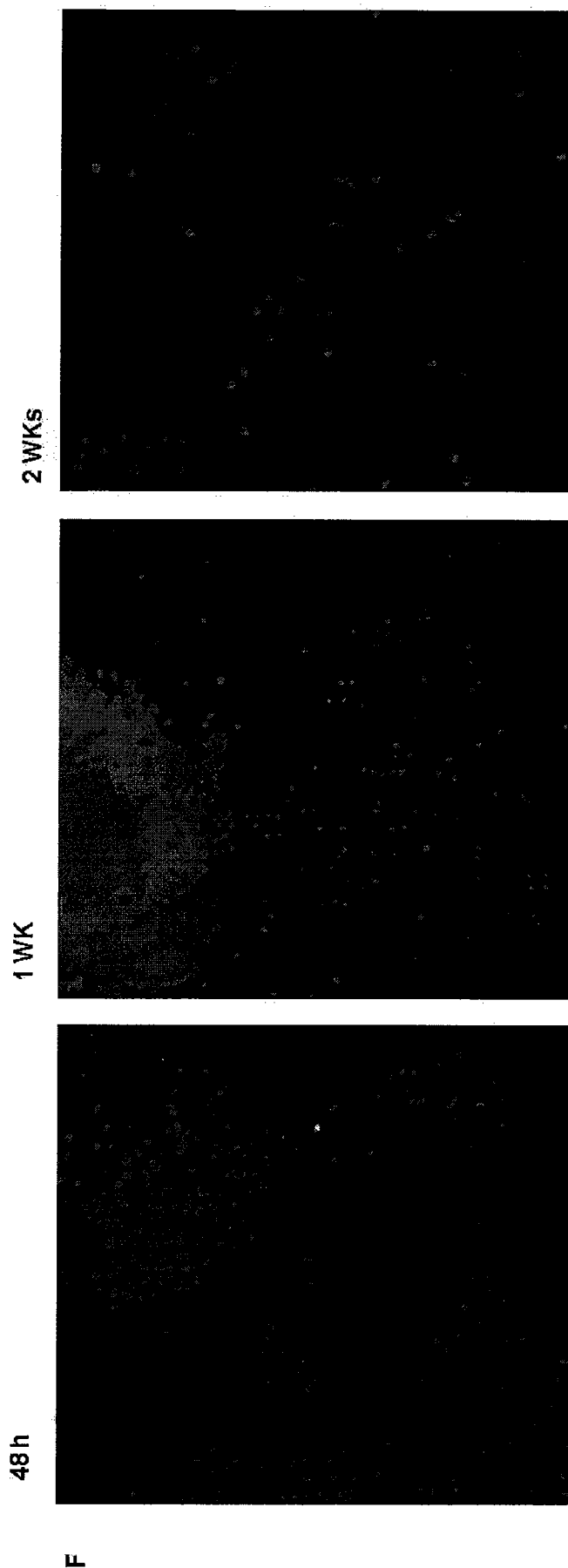


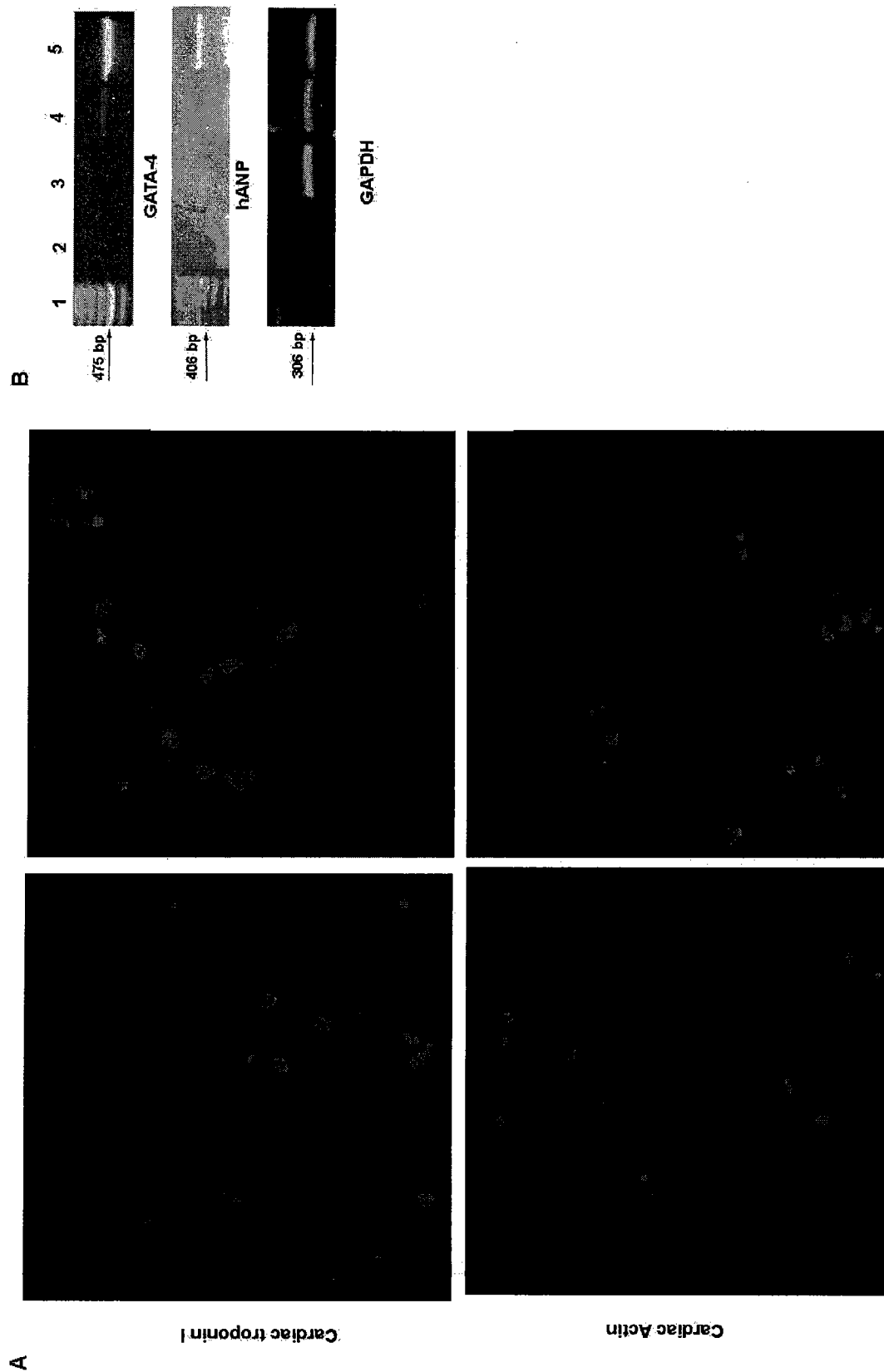
Figure 5. (Continued)



## Oligo and CD45

**Figure 5.** Time course analysis of neuronal and non-neuronal differentiation. (A) morphological (B) genetic and (C) phenotypic changes in MNC cultured in NCC and grown on chamber slides or six-well plates. (A) Phase contrast microscopy of MNC before and after 24 h, 1, 2 and 3 weeks in NCC demonstrates the formation of spherical structures and progressive development of cells with neuron-like projections. (B) RT-PCR analysis of OCT-4, nestin and positive control GAPDH-specific transcript expression. Lane orders for each gene transcript; lane 1, 100-bp ladder; lane 2, gene transcript-specific negative control, lane 3, 24 h MNC/NCC; lane 4, 1 week MNC/NCC; lane 5, 2 weeks MNC/NCC; lane 6, 3 weeks MNC/NCC and lane 7, MNC alone. (C) Confocal microscopy of NCC-cultured MNC at defined time points co-stained with the nuclear stain hoechst (blue) and either NF, GFAP or MAP2 (each red) demonstrating the progressive acquisition of neuronal-specific markers. Differential staining of neurons, glia and oligodendrocytes with time by dual staining with (D) MAP-2 (blue) and Tau (green) and lower images at 2 weeks are of the same field showing MAP-2 being confined to cell bodies and Tau to axons, (E) Pan-NF (blue) and GFAP (green) and (F) oligodendrocyte (blue) and CD45 (green). In (D-F) nuclei were stained with propidium iodide (PI) (red)





**Figure 6.** Analysis of cardiac differentiation. (A) phenotypic and (B) time course analysis of genetic changes in MNC cultured in CCC. (A) Differential expression of either cardiac Troponin I or cardiac Actin (both blue) without CD45 (green) after 1 week in culture. Nuclei were stained (red) with PI. (B) RT-PCR analysis of cardiac transcription factors, GATA-4 and hANP and positive control GAPDH-specific transcript expression. Lane orders for each gene transcript: lane 1, 100 bp ladder; lane 2, gene transcript-specific negative control; lane 3, MNC alone; lane 4, 24 h MNC/CCC; lane 5, 1 week MNC/CCC

## Discussion

Stem cell plasticity is a newly discovered phenomenon. Recently, cells with multi-developmental potentials have been identified in the bone marrow following *ex vivo* expansion<sup>30</sup>. In contrast, herein, four different types of pluripotent progenitor/stem cells were produced from MNC obtained from unmobilised peripheral adult human blood. In response to the addition of CR3/43 mAb to a variety of well-established culture conditions, HCC, NCC or CCC give rise to haematopoietic, undifferentiated and, subsequently, neuronal or cardiomyogenic progenitor cells. This occurs despite the fact that the source material can be derived from a single blood donor.

Human MNC cultured under HCC are capable of engrafting and differentiating into a variety of lymphohaematopoietic cell lineages in the NOD/SCID mouse model {manuscript in preparation}. In addition, such cells when infused into the myocardium of the *Rnu/Rnu* nude rat<sup>49</sup> engrafted and differentiated into fully mature human cardiomyocytes 1 week later {unpublished data}, thus further confirming the phenomenon of somatic cell plasticity<sup>2-5,50</sup>. Moreover, a significant proportion of MNC cultured in ES medium supplemented with CR3/43 mAb were converted into cells able to transcribe embryonic stem cells antigen, OCT-4; a transcription factor that is normally restricted in its expression to pluripotent cells<sup>51</sup>. These latter undifferentiated cells can be purified and are able to form EB-like structures in 'hanging drop' cell cultures following proliferation. Continued culturing of MNC in NCC gives rise to fully mature neurons, glia and oligodendrocytes upon extinguishing of nestin and OCT-4, that become capable of secreting a variety of neurotransmitters {unpublished data} – one of the *bona fide* characteristics of neurons. The *in vivo* functional utility of such cells in the reversal of Parkinson's disease in an animal model warrants investigation. Furthermore, the cardiomyogenic progenitor cells produced in response to culturing of MNC in CCC mechanically resemble beating embryoid bodies which appear to give rise to novel cells that are capable of synchronous beating rhythms in 'hanging drop' cell cultures (see supplementary data – Real-time movie B<sup>48</sup>). As mentioned, these cells were able to engraft and differentiate *in vivo* into mature cardiomyocytes. The fact that the cells can follow either a neuronal or cardiomyogenic fate by either culturing against gravity as 'hanging drops' or upright in a six-well plate, with or without artificially introduced extracellular matrix, is particularly noteworthy. This implicates the physical environment in directing the specialisation fate of a group of cells spatially organised in a certain configuration, and profoundly altering their developmental destiny, a notion that may be of importance in engineering and sculpturing human tissue.

The data presented in this report shows for the first time that the phenomenon of somatic cell plasticity is not exclusive to existing stem cells *in vivo* or to embryonic stem cells newly formed via nuclear transfer. Optionally, differentiated cells can also exhibit pluripotency *in vitro*, a notion that may redefine what is a differentiated or a stem cell state. Furthermore, as documented herein, CR3/43 mAb appears to facilitate de-differentiation in MNC<sup>32</sup>, while the immediate physical and biochemical surroundings of the cells cause their transdifferentiation or traversal of the differentiation barrier into new and multiple specialisation fates. In this manner, examples whereby signalling through MHC class II has influenced the cell physiology and behaviour include aggregation<sup>53</sup>, activation<sup>54,55</sup>, proliferation<sup>56-59</sup>, anergy<sup>53,60,61</sup> and apoptosis<sup>62,63</sup>. As to why MHC class II cross-linking profoundly alters cell specialisation fate, may be reflected by the differential association of protein kinase C isoforms with HLA-DR $\beta$  chains<sup>64</sup>. For example, alternate protein kinase C isoforms have been implicated in controlling differentiation of murine F9 embryonal carcinoma cells: one isoform promotes differentiation into parietal endoderm, whereas another stimulates the retrodifferentiation of such endodermal cells back into multipotent progenitors<sup>65</sup>.

The induction of stem cell-like plasticity in a heterogeneous population of leukocytes, predominantly comprising mature specialised cells, may have proceeded by a process of retrodifferentiation<sup>20-25</sup>. This mechanism behind the reprogramming of differentiation in a population of adult cells may be contentious but, nonetheless, may address the phenomena of somatic or stem cell plasticity in haematopoietic stem cells, including the recently described multipotent adult progenitor cells<sup>30</sup>. Irrespective of their origins, the ultimate functional utility of all stem cell types, whatever their source, remains to be determined in diseases where normal physiological functions of a variety of degenerate tissues need to be restored, either in autologous or allogeneic settings.

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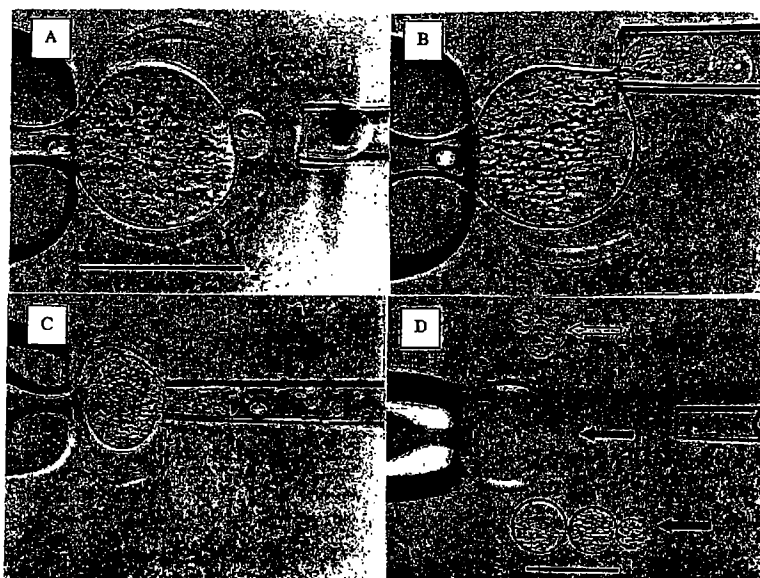
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(54) Title: PLURIPOTENT STEM CELLS DERIVED WITHOUT THE USE OF EMBRYOS OR FETAL TISSUE



(57) Abstract: This invention provides a method for deriving precursors to pluripotent non-embryonic stem (P-PNES) and pluripotent non-embryonic stem (PNES) cell lines. The present invention involves nuclear transfer of genetic material from a somatic cell into an enucleated, zona pellucida free human ooplastoid having a reduced amount of total cytoplasm. The present invention provides a new source for obtaining human and other animal pluripotent stem cells. The source utilizes as starting materials an oocyte and a somatic cell as the starting materials but does not require the use, creation and/or destruction of embryos or fetal tissue and does not in any way involve creating a cloned being. The oocyte never becomes fertilized and never develops into an embryo. Rather, portions of

the oocyte cytoplasm are extracted and combined with the nuclear material of individual mature somatic cells in a manner that precludes embryo formation. Murine, bovine, and human examples of the procedure are demonstrated. Subsequently, the newly constructed P-PNES cells are cultured *in vitro* and give rise to PNES cells and cell colonies. Methods are described for culturing the P-PNES cells to yield purified PNES cells which have the ability to differentiate into cells derived from mesoderm, endoderm, and ectoderm germ layers. Methods are described for maintaining and proliferating PNES cells in culture in an undifferentiated state. Methods and results are described for analysis and validation of pluripotency of PNES cells including cell morphology, cell surface makers, pluripotent tumor development in SCID mouse, karyotyping, immortality in *in vitro* culture.

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## **PLURIPOTENT STEM CELLS DERIVED WITHOUT THE USE OF EMBRYOS OR FETAL TISSUE**

This application claims the benefit of U.S. Patent Application Serial No.  
5 10/026,420, filed December 18, 2001, which is hereby incorporated by reference in its entirety.

### **TECHNICAL FIELD OF THE INVENTION**

10 This invention relates to the creation, production, maintenance, growth and application of human and animal pluripotent stem cells that have been created without the use and/or destruction of embryos (whether naturally derived or created via a cloning process) and without the need for fetal tissue, or "pluripotent non-embryonic/non-fetal tissue derived stem cells" (hereinafter, "PNES," and reference to "PNES" throughout this filing shall incorporate  
15 both human and animal PNES cells unless otherwise indicated). More specifically this invention provides (a) a method for deriving cells which are precursors to PNES cells ("P-PNES cells") via the nuclear transfer of genetic material from a somatic cell into an enucleated, zona pellucida free portion of an ooplast having a reduced amount of total ooplasm (referred to as an "ooplastoid"), and a method for keeping those P-PNES cells from clumping or gathering into a  
20 cell mass, (b) methods of culturing and converting the P-PNES cells into actual PNES cells and PNES cell lines and for methods/techniques for establishing the characteristics (including immortality and pluripotency) of those PNES cells, (c) methods for maintaining and proliferating the PNES cells and PNES cell lines in an undifferentiated state, (d) methods and techniques for directing those PNES cells to become multipotent/adult stem cells including, but not limited to,  
25 blood stem cells, neural stem cells, liver stem cells, and other stem cells and/or Specific Differentiated Cells, (e) methods for directing those multipotent/adult stem cells to become more specialized (differentiated) cells which no longer have the ability to differentiate, including, but not limited to, sertoli cells, endothelial cells, endothelial cells, granulosa epithelial, neurons, pancreatic islet cells, epidermal cells, epithelial cells, hepatocytes, hair follicle cells,  
30 keratinocytes, hematopoietic cells, melanocytes, chondrocytes, lymphocytes (B and T lymphocytes), erythrocytes, macrophages, monocytes, mononuclear cells, fibroblasts, cardiac muscle cells, and other muscle cells, etc. and (f) the use of those P-PNES, PNES, multipotent/adult stem cells, and Specific Differentiated Cells and derivatives thereof for scientific and therapeutic purposes. The scientific and therapeutic applications include, but are  
35 not limited to, use in (a) scientific discovery and research involving cellular development and genetic research, (b) drug development and discovery, (c) gene therapy, and (d) treatment of

diseases and disorders including, but not limited to, (i) tissue/cellular regeneration and replacement therapies and applications, (ii) immune system disorders, (iii) blood disorders, (iv) cancer, and a variety of other diseases and disorders.

5

## BACKGROUND OF THE INVENTION

“Pluripotent stem cells” are undifferentiated cells that have the potential to divide *in vitro* for a long period of time (greater than one year) and have the unique ability to differentiate into (and therefore are a potential source for) cells derived from all three embryonic germ layers - - endoderm, mesoderm and ectoderm. This ability to differentiate into all three embryonic germ layers is referred to as “pluripotency.” The significant scientific and therapeutic potential of these cells, particularly because of their pluripotent nature, is monumental, and includes, but is not limited to, use in (a) scientific discovery and research involving cellular development and genetic research, (b) drug development and discovery, (c) gene therapy, and (d) tissue/cellular regeneration and replacement therapies and applications. It is also important to note that pluripotent stem cells do not have the ability to become an embryo or complete human or animal organism. In other words, these cells can differentiate into every cell found in a mature animal or human, but not the animal or human itself.

To date, there have been created two categories of pluripotent stem cells. “Embryonic stem cells,” as defined by the scientific community, are pluripotent stem cells that are derived directly from an embryo (to date, these embryos have been obtained via a naturally fertilized egg or via cloning). “Embryonic germ” cells are pluripotent stem cells that are derived directly from the fetal tissue of aborted fetuses. For purposes of simplicity, embryonic stem cells and embryonic germ cells will be collectively referred to as “ES” cells unless otherwise indicated. There are also reports that cells with some characteristics of human pluripotent ES cells may be created using a combination of human cells and oocytes from other animal species. Each of these current methods for creating pluripotent ES cells is described in more detail here.

As mentioned, two techniques are employed to create ES via the destruction of viable embryos. The first method utilizing human embryos was under US Patents 5,843,780 and 6,200,806, pursuant to which the inventor, Dr. Thompson, first derived a human ES cell line from the inner cell mass of normal human embryos in the blastocyst stage (United States Patent No. 6,200,806 and Thompson, J.A. et al. *Science*, 282:1145-7, 1998). The blastocyst is formed approximately five days after fertilization of an oocyte by a sperm cell. The blastocyst stage embryos were donated by couples undergoing in vitro fertilization therapy. The ES stem cells produced by Thompson could proliferate *in vitro*, in an undifferentiated state, for more than one year if they were grown on a fibroblast feeder layer. These cells retained the ability to



differentiate into endoderm, mesoderm or ectoderm lineage cells over this time period, thus displaying the characteristic of pluripotency. As a result of Dr. Thompson's process/method, the human embryos were destroyed. The second method for creating pluripotent ES cells which also involves the destruction of embryos utilizes the technique of somatic cell nuclear transfer (SCNT) in a practice pursuant to which an embryo is created via cloning, and then destroyed in the process that obtained the pluripotent ES cells from that embryo. The potential of this technique was demonstrated by Campbell and Wilmut using farm animal species wherein individual animals were cloned (See United States Patent No. 6,147,276 and 6,252,133). In this technique the nucleus is removed from a normal egg, thus removing the genetic material. Next, a donor diploid somatic cell is placed next to the enucleated egg and the two cells are fused. The fused cell has the potential to develop into a viable embryo which may theoretically then be sacrificed in order to remove that portion of the embryo containing the stem cell producing inner cell mass. The use of this method in humans would thus involve creating a cloned embryo autologous to the donor of the somatic cells followed by the destruction of the human embryo.

Pursuant to another reported method that may create pluripotent ES cells, the nucleus of a human cell is transplanted into an entire enucleated animal oocyte of a species different from the donor cell (referred to herein as animal stem cell nuclear transfer, or "ASCNT"). See U.S. Patent Application No. 20010012513 (2001). The resultant chimeric cells are potentially used for the production of pluripotent ES cells, in particular human-like pluripotent ES cells. One disadvantage of this technique is that these chimeric cells may contain unknown non-human viruses and still contain the mitochondria of the animal species and thus there would be substantial risks of immune rejections if such cells were used in cell transplantation therapies.

The final reported technique for obtaining pluripotent ES cells requires the dissection of 8-11 week old aborted human fetuses. Under this method, human primordial embryonic germ cells are extracted from the gonadal ridges and mesenteries of aborted fetuses (U.S. Patent 6,090,622 and M. J. Shamblott *et al. Proc. Natl. Acad. Sci. USA*, 95:13726-13731, 1998). The human pluripotent ES cells produced in this manner were dependent on the presence of certain growth factors and ligands in the culture medium such as leukemia inhibitory factor (LIF), basic fibroblast growth factor and forskolin. In addition, the ES cells derived from human primordial embryonic germ cells differed slightly in cell morphology and surface marker expression from those derived from 5 day old blastocysts. These cells are believed to be pluripotent because immunohistochemical analysis of the embryoid bodies that form in cultures show antibody staining that is consistent with the presence of cells derived from the three embryonic germ layers.

Pluripotent stem cells (which include pluripotent ES cells) can be differentiated from

“multipotent stem cells.” A multipotent stem cell has the ability to differentiate into some but not all of the cells derived from all three germ layers. For example, a “blood stem cell” is thought to be multipotent because it has the ability to differentiate into all types of specific blood cells, but it is unlikely that they can differentiate into all cells of a given animal or human. Multipotent stem cells exist in vivo (for example, blood stem cells can be found in bone marrow and the blood of adult animals and humans), and such *in vivo* cells also referred to as “adult stem cells.” In addition, multipotent stem cells can be created by directing pluripotent stem cells to become certain multipotent stem cells. (The term “multipotent/adult stem cell(s)” will be used to describe multipotent stem cells whether the source is *in vivo* or some other methodology or technique.) While not offering the same breadth of promise as pluripotent stem cells, multipotent/adult stem cells have a great deal of promise in research and in the area of therapeutic applications. For example, multipotent/adult stem cells have already been used in humans in attempts to treat certain blood, neural and cancer diseases.

It is also helpful to distinguish between pluripotent stem cells and “totipotent stem cells.” Totipotent stem cells have the ability to not only differentiate into cells derived from all three germ layers just as pluripotent stem cells can, but they also have the ability to grow into a complete human being or animal, something which pluripotent stem cells such as pluripotent ES cells cannot accomplish.

Unfortunately, to date, pluripotent ES cells can only be derived from these sometimes-controversial sources - - embryos (created naturally or via cloning), fetal tissue and via the mixing of materials of multiple species. The controversy surrounding the sources for such cells, according to many leading scientists and public and private organizations including the NIH, has greatly compromised and slowed the study of such cells and their application. In addition to the issues surrounding the sources of pluripotent ES cells, the other major shortcomings of some or all of the pluripotent ES cells created via current techniques include the following: (a) the use of current human ES lines obtained from the destruction of human embryos (e.g., those cell lines created by Dr. Thompson) is inappropriate according to the NIH because the cells have been exposed to animal cells (i.e., grown on mouse feeder layers); and (b) use of embryonic and fetal tissue derived stem cells may have limited application in humans because the genetic make-up of the resulting pluripotent ES cells will be different than that of any particular patient, causing issues of rejection by the immune system in the case, for example, of cellular or tissue transplants. Research and applications of multipotent/adult stem cells has also been hindered by various factors including (a) not all human adult stem cells have been isolated in tissue, (b) these cells are very difficult to isolate and purify, (c) they come in very minute quantities from in vivo sources and limited numbers are being created via the manipulation of pluripotent ES cells, (d)

they do not last as long as pluripotent cells in vitro, (e) they are difficult to grow quickly enough to be used for acute disorders, (f) they can't be used to study early cell development, and (g) while they may be able to differentiate into other cells, they have not been shown to be pluripotent.

5 All of these major shortcomings have created a great demand for (a) methods of creating pluripotent ES cells without the use of embryos (naturally created or created via cloning) or fetal material and without the need to involve mixing of species cells or cell materials, (b) the ability to create pluripotent ES cells specific to a particular patient or disease population, a new and more plentiful and useful, and (c) a more plentiful source for multipotent/adult stem cells than is  
10 currently available.

### OBJECTS OF THE INVENTION

All of the objects set forth herein apply to humans and animals. "Animals" shall include ovine, bovine, porcine, equine, murine, and other laboratory, farm and/or household animals.

15 The objects of this invention include the following:

It is an object of the present invention to provide for a method for the creation of "ooplastoids," which are enucleated, membraned, zona-pellucida free ooplasts and which result from the splitting of an enucleated oocyte into 2 to 6 portions.

It is an object of the present invention to provide ooplastoids.

20 It is an object of the present invention to provide a procedure for reprogramming a somatic cell nucleus using an "ooplastoid."

It is an object of the present invention to provide a method for making ooplastoids that can be combined with somatic cells or somatic cell nuclei to give rise to precursor cells known as nascent cells which give rise to pluripotent non-embryonic/non-fetal tissue derived stem cells  
25 that are pluripotent and can proliferate in culture indefinitely and in an undifferentiated state. These precursor cells are referred to as "P-PNES" or "P-PNES cells."

It is an object of the present invention to provide P-PNES cells.

It is an object of the present invention to provide P-PNES cells via nuclear transfer through combining an ooplastoid and a somatic cell or somatic cell nucleus.

30 It is a further object of the present invention to provide for a method for keeping P-PNES and PNES cells from clustering, grouping or contracting during in vitro culture.

It is further object of the present invention to culture and direct P-PNES cells into pluripotent non-embryonic/non-fetal tissue derived stem cells that are pluripotent and can proliferate in culture indefinitely and in an undifferentiated state (as indicated, these cells are  
35 referred to as "PNES" or "PNES cells" or "PNES cell lines").

It is an object of this invention to provide P-PNES and PNES cells that can be identified, isolated and purified.

It is an object of this invention to provide for methods of identifying, isolating and purifying P-PNES cells and PNES cells.

5 It is a further object of this invention to provide PNES that can proliferate in culture in an undifferentiated state for more than one year and wherein the cells remain euploid..

It is another object of the present invention to provide for methods to maintain PNES cells in culture in an undifferentiated state.

10 It is a further object of the present invention to provide for methods of growing/proliferating PNES cells in culture.

It is an object of the present invention to provide PNES cells that retain the potential to differentiate into tissues derived from all three germ layers: endoderm, mesoderm, and ectoderm.

15 It is an object of the present invention to create P-PNES and PNES cells/cell lines that are not totipotent and are not embryogenic (e.g., human PNES cells can not develop into a human being if implanted in a woman's uterus).

20 It is an object of this invention to provide methods for creating P-PNES and PNES cells that are autologous to the source/donor of the somatic cell involved in the nuclear transfer and as a result it is the object of this invention to provide P-PNES and PNES cell lines that share the genetic make-up and characteristics of any specific/individual animal or human being or specific population (e.g. disease populations, racial populations, etc.).

25 It is an object of this invention to provide PNES cells that are autologous to the source/donor of the somatic cell involved in the nuclear transfer and as a result it is the object of this invention to provide P-PNES and PNES cell lines that share the genetic make-up and characteristics of any specific/individual animal or human being or specific population (e.g. disease populations, racial populations, etc.).

30 It is an object of the present invention to provide PNES cell lines which exhibit the same characteristics and properties of pluripotent ES cells (e.g., pluripotency, ability to remain undifferentiated in culture for more than one year, etc.), including characteristic and properties related to cell morphology, karyotypes, cell markers, and other tests/characteristics familiar to and accepted by the stem cell scientific community.

It is a further object of the current invention to provide for methods, tests and proofs utilized to prove the properties of PNES cells, including but not limited to tests to show/prove characteristics of pluripotency, cell morphology, karyotypes, and cell markers.

35 It is an object of this invention to provide for methods of utilizing PNES cells and their

derivatives in scientific and therapeutic applications including, but not limited to, (a) scientific discovery and research involving cellular development and genetic research, (b) drug development and discovery (e.g., screening for efficacy and toxicity of certain drug candidates and chemicals), (c) gene therapy (e.g., as a delivery device for gene therapy), and (d) treatment of diseases and disorders including, but not limited to, Parkinson's, Alzheimer's, Huntington's, Ty Sachs, Gauchers, spinal cord injury, stroke, burns and other skin damage, heart disease, diabetes, Lupus, osteoarthritis, liver diseases, hormone disorders, kidney disease, leukemia, lymphoma, multiple sclerosis, rheumatoid arthritis, Duchenne's Muscular Dystrophy, Ontogenesis Imperfecta, birth defects, infertility, pregnancy loss, and other cancers, degenerative and other diseases and disorders.

It is a further object of this invention to provide for methods to direct PNES cells to differentiate into multipotent/adult stem cells derived from all three germ layers, including, but not exclusively, blood stem cells, neural stem cells, liver stem cells, and pancreatic stem cells.

It is a further object of this invention to provide/create multipotent/adult stem cells (derived from PNES) including, but not limited to, blood stem cells, neural stem cells, liver blood cells, and pancreatic stem cells.

It is a further object of this invention to provide for methods of identifying, isolating and purifying multipotent/adult stem cells derived from PNES, including, but not limited to, blood stem cells, neural stem cells, liver stem cells, and pancreatic stem cells.

It is a further object of this invention to provide for methods of proliferating multipotent/adult stem cells derived from PNES cells (including, but not limited to, blood stem cells, neural stem cells, liver stem cells, and pancreatic stem cells) in culture in an undifferentiated state.

It is a further object of the current invention to provide for methods, tests and proofs utilized to prove the properties of multipotent/adult stem cells derived from PNES, including but not limited to tests to show/prove characteristics of multipotency, cell morphology, karyotypes, and cell markers.

It is an object of this invention to provide for methods of utilizing multipotent/adult stem cells derived from PNES, and their derivatives, in scientific and therapeutic applications including, but not limited to, (a) scientific discovery and research involving cellular development and genetic research, (b) drug development and discovery (e.g., screening for efficacy and toxicity of certain drug candidates and chemicals), (c) gene therapy (e.g., as a delivery device for gene therapy), and (d) treatment of diseases and disorders including, but not limited to, Parkinson's, Alzheimer's, Huntington's, Ty Sachs, Gauchers, spinal cord injury, stroke, burns and

other skin damage, heart disease, diabetes, Lupus, osteoarthritis, liver diseases, hormone disorders, kidney disease, leukemia, lymphoma, multiple sclerosis, rheumatoid arthritis, Duchenne's Musclar Dystrophy, Ontogenesis Imperfecto, birth defects, infertility, pregnancy loss, and other cancers, degenerative and other diseases and disorders.

5 It is a further object of this invention to provide for methods to direct multipotent/adult stem cells (derived from PNES cells) to differentiate into specific cell types derived from all three germ layers which have no capacity for further differentiation since they represent terminal differentiation stage (e.g., sertoli cells, endothelial cells, endothelial cells, granulosa epithelial, neurons, pancreatic islet cells, epidermal cells, epithelial cells, hepatocytes, hair follicle cells, 10 keratinocytes, hematopoietic cells, melanocytes, chondrocytes, lymphocytes (B and T lymphocytes), erythrocytes, macrophages, monocytes, mononuclear cells, fibroblasts, cardiac muscle cells, and other muscle cells, etc. and hereinafter, referred to as "Specific Differentiated Cells"),

It is a further object of this invention to provide Specific Cell types which represent cells 15 derived from all three germ layers and which do not have any differentiation abilities, including, but not limited to, sertoli cells, endothelial cells, granulosa epithelial, neurons, pancreatic islet cells, epidermal cells, epithelial cells, hepatocytes, hair follicle cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, lymphocytes (B and T lymphocytes), erythrocytes, macrophages, monocytes, mononuclear cells, fibroblasts, cardiac muscle cells, and 20 other muscle cells, etc.

It is a further object of this invention to provide for methods of identifying, isolating and purifying Specific Differentiated Cells including, but not limited to, sertoli cells, endothelial cells, granulosa epithelial, neurons, pancreatic islet cells, epidermal cells, epithelial cells, hepatocytes, hair follicle cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, 25 lymphocytes (B and T lymphocytes), erythrocytes, macrophages, monocytes, mononuclear cells, fibroblasts, cardiac muscle cells, and other muscle cells, etc.

It is a further object of this invention to provide for methods of proliferating Specific Differentiated Cells including, but not limited to sertoli cells, endothelial cells, granulosa epithelial, neurons, pancreatic islet cells, epidermal cells, epithelial cells, hepatocytes, hair 30 follicle cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, lymphocytes (B and T lymphocytes), erythrocytes, macrophages, monocytes, mononuclear cells, fibroblasts, cardiac muscle cells, and other muscle cells, etc.

It is a further object of the current invention to provide for methods, tests and proofs 35 utilized to prove the properties of Specific Differentiated Cells, including but not limited to tests to show/prove characteristics of cell morphology, karyotypes, and cell markers.

It is a further object of this invention to provide for methods of utilizing Specific Differentiated Cells and their derivatives in scientific and therapeutic applications including, but not limited to, (a) scientific discovery and research involving cellular development and genetic research, (b) drug development and discovery (e.g., screening for efficacy and toxicity of certain drug candidates and chemicals), (c) gene therapy (e.g., as a delivery device for gene therapy), and (d) treatment of diseases and disorders including, but not limited to, Parkinson's, Alzheimer's, Huntington's, Ty Sachs, Gauchers, spinal cord injury, stroke, burns and other skin damage, heart disease, diabetes, Lupus, osteoarthritis, liver diseases, hormone disorders, kidney disease, leukemia, lymphoma, multiple sclerosis, rheumatoid arthritis, Duchenne's Musclar Dystrophy, Ontogenesis Imperfecto, birth defects, infertility, pregnancy loss, and other cancers, degenerative and other diseases and disorders.

### SUMMARY OF THE INVENTION

The present invention provides a new source for obtaining pluripotent stem (PNES) cells. The process/method of creating PNES cells utilizes an oocyte and a somatic cell as the starting materials but does not require the use, creation and/or destruction of embryos or fetal tissue and does not in any way involve creating a cloned human or animal. This invention provides a method for deriving nascent cells which are precursors of PNES cells via nuclear transfer of genetic material from a somatic cell into an enucleated, zona pellucida free ooplast having a reduced amount of total cytoplasm. The oocyte used in this procedure never becomes fertilized and never develops into an embryo. Rather, portions of the oocyte cytoplasm are obtained and combined with the nuclear material of individual mature somatic cells in a manner that precludes embryo formation. Instead, the cells formed are precursors to PNES, or "P-PNES." Subsequently, the newly constructed P-PNES cells are cultured *in vitro* and give rise to PNES cells and cell colonies. More specifically, this invention also provides (a) methods of isolating, identifying, and culturing the P-PNES cells to yield purified PNES cells which have the ability to differentiate into cells derived from mesoderm, endoderm, and ectoderm germ layers, (b) methods for isolating, purifying, identifying and maintaining and proliferating PNES cells in culture in an undifferentiated state for more than one year, and (c) the use of those PNES cells and derivatives thereof for scientific and therapeutic purposes. These applications include, but are not limited to, use of PNES cells and derivatives thereof in (a) scientific discovery and research involving cellular development and genetic research, (b) drug development and discovery, (c) gene therapy, and (d) tissue/cellular regeneration and replacement therapies and applications, and treatment for other diseases and disorders).

The current invention also provides for methods for directing pluripotent PNES cells to become multipotent/adult stem cells (referred to herein as ASC's) that individually have the ability to differentiate into some but not all of the cells derived from all three germ layers. For example, ASC's would include, but not exclusively, blood stem cells, which have the ability to differentiate into some, but not all, cells derived from all three germ layers. More specifically, this invention also provides (a) methods of culturing and directing PNES to yield purified ASC's which have the ability to differentiate into some but not all cells derived from mesoderm, endoderm, and ectoderm germ layers, (b) methods for isolating, purifying, identifying and maintaining and proliferating ASC's in culture in an undifferentiated state, and (c) the use of those ASC's and derivatives thereof for scientific and therapeutic purposes. These applications include, but are not limited to, use of ASC's and derivatives thereof in (a) scientific discovery and research involving cellular development and genetic research, (b) drug development and discovery, (c) gene therapy, and (d) tissue/cellular regeneration and replacement therapies and applications, and treatment for other diseases and disorders).

In addition to the above, the current invention provides for methods of directing ASC's to become Specific Differentiated Cells which no longer have the ability to differentiate, or "Specific Differentiated Cells" sertoli cells, endothelial cells, granulosa epithelial, neurons, pancreatic islet cells, epidermal cells, epithelial cells, hepatocytes, hair follicle cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, lymphocytes (B and T lymphocytes), erythrocytes, macrophages, monocytes, mononuclear cells, fibroblasts, cardiac muscle cells, and other muscle cells, etc. More specifically, this invention also provides (a) methods of culturing and directing ASC's to yield purified Specific Differentiated Cells which no longer have the ability to differentiate, (b) the use of those Specific Differentiated Cells and derivatives thereof such as sertoli cells, endothelial cells, granulosa epithelial cells, neurons, pancreatic islet cells, epidermal cells, epithelial cells, hepatocytes, hair follicle cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, lymphocytes (B and T lymphocytes), erythrocytes, macrophages, monocytes, mononuclear cells, fibroblasts, cardiac muscle cells, and other muscle cells, etc for scientific and therapeutic purposes. These applications include, but are not limited to, use of Specific Differentiated Cells and derivatives thereof in (a) scientific discovery and research involving cellular development and genetic research, (b) drug development and discovery, (c) gene therapy, and (d) tissue/cellular regeneration and replacement therapies and applications, and treatment for other diseases and disorders).

In accordance of the above objects and others, the present invention is related in part to a



purified preparation of pluripotent non-embryonic stem cells, which (i) is capable of proliferating in an *in vitro* culture for more than one year; (ii) maintains a karyotype in which the cells are euploid and are not altered through culture; (iii) maintains the potential to differentiate into cell types derived from the endoderm, mesoderm and ectoderm lineages throughout the culture, and  
5 (iv) is inhibited from differentiation when cultured on fibroblast feeder layers.

More particularly, the present invention is directed to pluripotent non-embryonic stem cells that display the following characteristics: the cells are negative for expression of the SSEA-1 marker; the cells express elevated alkaline phosphatase activity; the cells are positive for  
10 expression of the TRA-1-81 marker and the TRA-1-60 marker; the cells are positive for expression of the CCA-3 and CCA-4 Markers; and the cells are able to differentiate into cells derived from mesoderm, endoderm and ectoderm germ layers when the cells are injected into a SCID mouse.

15 This invention is further related to pluripotent non-embryonic stem cells and methods of producing them in which the cells are human, or non-human animal such as from the following animals: of dog, cat, mouse, rat, cow, pig, sheep, goat, horse, buffalo, llama, ferret, guinea pig, rabbit and any other mammalian species.

20 The invention is further related to a purified preparation of pluripotent non-embryonic stem cells, which (i) is capable of proliferating in an *in vitro* culture for an indefinite period; (ii) maintains a karyotype in which the cells are euploid and are not altered through culture; and (iii) maintains the potential to differentiate into cells types derived from the endoderm, mesoderm and ectoderm lineages throughout the culture.

25 The invention is further related to stem cells which do not originate from a fertilized egg, but which originates from the combination of a somatic cell nucleus and an enucleated ooplastoid.

30 The invention is further related to stem cells which do not originate from fetal tissue, but which originates from the combination of a somatic cell nucleus and an enucleated ooplastoid.

The present invention provides stem cells which do not originate from a fertilized egg or from fetal tissue, but which originates from the combination of a somatic cell nucleus and an  
35 enucleated ooplast or super-ooplast.

The invention is further related to stem cell which is produced by the method of (i) contacting a desired somatic cell or somatic cell nucleus with an ooplastoid, wherein the ooplastoid is derived from an enucleated oocyte; (ii) combining the somatic cell or somatic cell nucleus with an ooplastoid to create a nascent cell, and (iii) culturing the nascent cell to obtain pluripotent non-embryonic stem cells.

The invention is further related to a nascent cell produced from the combination of a somatic cell nucleus and an enucleated zona pellucida free ooplastoid.

In accordance with the above objects and others, the present invention provides method of producing pluripotent, non-embryonic stem cells comprising the following steps: (i) contacting a desired somatic cell or somatic cell nucleus with an ooplastoid, wherein the ooplastoid is derived from an enucleated oocyte; (ii) combining the somatic cell or somatic cell nucleus with an ooplastoid to create a nascent cell; (iii) activating the nascent cell; and (iv) culturing the nascent cell to obtain pluripotent non-embryonic stem cells.

In another embodiment of the present invention, the ooplastoid used in the method to generate pluripotent non-embryonic stem cells contains from about 10% to about 100% of the cytoplasmic volume of a mature oocyte.

In another embodiment of the present invention, the ooplastoid used in the method to generate pluripotent non-embryonic stem cells contains less than about 50% of the cytoplasmic volume of a mature oocyte.

In one embodiment of the present invention, the ooplastoid used in the method to generate pluripotent non-embryonic stem cells contains from about 17% to about 33% of the cytoplasmic volume of a mature oocyte.

In particular embodiments, the present invention is related to a method of producing pluripotent, non-embryonic stem cells wherein the somatic cell or somatic cell nucleus is a mature cell or where the somatic cell is an epithelial cell, lymphocyte or fibroblast.

In particular embodiments, the present invention is related to methods of producing pluripotent, non-embryonic stem cells where the somatic cell or somatic cell nucleus is combined

with an ooplastoid to create a nascent cell by intracytoplasmic injection of the somatic cell nucleus into the zona free reduced volume ooplastoid; or where the somatic cell or somatic cell nucleus is combined with an ooplastoid to create a nascent cell by involves fusion induced by electrodes that are introduced directly into the culture dish and electrical pulses administered to the couplets immediately following micromanipulation; or where the somatic cell or somatic cell nucleus is combined with an ooplastoid to create a nascent cell by fusion in an electric field via electroporation; or fusion in a fusion chamber.

In particular embodiments, the present invention is related to methods of producing pluripotent non-embryonic stem cells comprising the following steps: (i) contacting one or more desired somatic cells or somatic cell nuclei with a super-ooplast derived from one or more enucleated zona pellucida free oocytes; (ii) dividing said super-ooplast into single nucleus containing nascent cells; (iii) activating the nascent cells; and (iv) culturing the nascent cells to obtain pluripotent non-embryonic stem cells.

In particular embodiments, the present invention is related to methods of producing pluripotent non-embryonic stem cells through using an enucleated zona pellucida free super-ooplast that comprises more than 100% of the cytoplasmic volume of a single egg and where the super-ooplast containing nuclei is partitioned into separate single nuclei containing nascent cells.

The present invention provides stem cells which are produced by the method of (i) contacting a desired somatic cell or somatic cell nucleus with an ooplastoid, wherein said ooplastoid is derived from an enucleated oocyte; (ii) combining said somatic cell or somatic cell nucleus with said ooplastoid to create a nascent cell, and (iii) culturing said nascent cell to obtain pluripotent non-embryonic stem cells.

The present invention provides a method of producing pluripotent non-embryonic stem cells comprising the following steps: (i) contacting a desired somatic cell or somatic cell nucleus with an ooplastoid, wherein the ooplastoid is derived from an enucleated oocyte; (ii) combining the somatic cell or somatic cell nucleus with the ooplastoid to create a nascent cell; and (iii) culturing the nascent cell to obtain pluripotent non-embryonic stem cells.

The present invention provides a method of producing pluripotent non-embryonic stem cells comprising the following steps: (i) contacting more than one desired somatic cells or somatic cell nuclei with an enucleated oocyte; (ii) dividing the oocyte somatic cell or oocyte

somatic cell nuclei pairs into nascent cells, wherein each of the nascent cells contains a single nucleus; (iii) activating the nascent cells; and (iv) culturing the nascent cells to obtain pluripotent non-embryonic stem cells.

5           The present invention provides a method of producing pluripotent non-embryonic stem cells, wherein the cells are cultured on feeder layers comprising fibroblasts.

          According to the present invention, the somatic cell or somatic cell nucleus used to produce nascent cells may be genetically modified prior to being used to generate pluripotent  
10 non-embryonic stem cells.

          In particular embodiments, the present invention is related to methods of producing an ooplastoid comprising the following steps: (i) harvesting an oocyte from a female;  
15 (ii) maturing said oocyte to metaphase II; (iii) breaching or removing the zona pelucida of the metaphase II oocyte; (iv) enucleating the oocyte by removing the polar body and nuclear DNA of the oocyte through the breach of the zona pelucida or by oocyte partitioning; and (v) aspirating and pinching off an ooplastoid from the enucleated oocyte.

20           In particular embodiments, the zona pelucida is breached or removed using a chemical agent or using mechanical action.

          In particular embodiments, the ooplastoid has from about 10% to about 100% of the volume from the original oocyte. In other embodiments, the ooplastoid has from about 15% to  
25 about 49% of the volume from the original oocyte. In a further embodiment, the ooplastoid has from about 17% to about 33% of the volume from the original oocyte.

#### TERMS AND DEFINITIONS

30           The following terms are employed in the description of our invention:

Activation -- refers to any materials and methods useful for stimulating a cell to divide.

Adult Stem Cells or "ASC's" -- are certain cells found in vivo that are believed to be multipotent in nature. Use of the term "ASC's" refers to adult stem cells and multipotent stem  
cells.

35           Animals -- non-human animal as used herein will be understood to include all vertebrate

animals, except humans.

Autologous -- refers to cells expressing the same major histocompatibility antigens (MHC) as the donor/source of the somatic cell used in the nuclear transfer process.

5 Cell -- the term cell can refer to an oocyte, nascent cell, ES cell, an EC cell, a PNES cell, a P-PNES cell, a somatic cell or an early stage embryo.

Conditioned Growth Medium -- refers to a growth medium that is further supplemented by factors derived from media obtained from cultures of feeder cells on which human PNES cells can be cultured.

10 Connective Tissue -- connective tissue includes bone, cartilage, ligament, tendon, stroma and muscle.

Cryopreserved -- the terms cryopreserving or cryopreserved as used herein refer to freezing a cell of the invention.

Enucleated -- describes an object/cell from which the nucleus has been removed.

15 ES Cells -- ES cells include embryonic stem cells and embryonic germ cells, and are believed to express the following characteristics: (i) the ability to divide in culture for an unlimited time and in an undifferentiated state, (ii) maintenance of a normal diploid karyotype, and (iii) pluripotency. Pluripotent ES cells are currently derived from embryos (naturally or via cloning) and/or fetal tissue as primary sources.

20 Euploidy -- the state of karyotype comprised to a normal number of non-altered chromosomes (e.g., for humans, 46).

Growth Medium -- growth medium means a suitable medium capable of supporting cell growth.

GV -- gastro-vesicular stage of Metaphase I maturation stage.

25 Immortality -- Immortal cells are capable of continuous indefinite replication *in vitro*. As a practical matter, immortality is measured by observing continued proliferation of cells for longer than one year in culture.

Karyotype -- a normal karyotype means that all chromosomes normally characteristic of the species are present and have not been noticeably altered.

30 Maturation Period -- the time period beginning with aspiration of the immature oocyte from either human or animal ovarian follicles and including the time spent maturing the oocytes in a maturation medium prior and lasting until the oocyte attains a certain maturation endpoint, such as metaphase II, but not limited to metaphase II. The maturation endpoints relevant to the present invention include germinal vesicle stage (P1) or (GV) metaphase I (M1), metaphase II (MII), and post-activation oocytes.

35 Multipotent Stem Cells -- these are stem cells that are found in mature animals/humans

and which are believed to be capable of differentiating into cells derived from some, but not all, embryonic germ layers. Use of the term "ASC's" refers to adult stem cells and multipotent stem cells.

5 Metaphase I Immature Oocytes -- refers to the stage of development known as Metaphase I of meiosis.

Nascent Cell -- the nascent cell is produced as a result of the fusion or injection of an individual somatic cell or cell nucleus with an ooplastoid. The P-PNES described herein are considered examples of nascent cells.

10 Oocyte -- the egg cell, a specialized cell that carries one half the normal number of chromosomes (haploid) and is surrounded a thick layer of glycoproteins and extracellular matrix material called the zona pellucida. In humans, the oocyte carries 23 chromosomes.

Oocytoid -- Oocytoids arise after multiple nuclei are inserted or fused into an ooplast or super-ooplast, and by fragmenting such multinucleated ooplasts or super-ooplasts into single nucleus containing nascent cells (oocytoids).

15 Ooplasts -- Ooplasts result from the enucleation of an oocyte. Ooplasts are enucleated, plasma-membrane enclosed, zona pellucida intact or zona pellucida free oocytes.

Super-ooplasts - result from the fusion of two or more ooplasts or (enucleated oocytes). Super-ooplasts of greater than 100% of the volume of a single oocyte may also be created by fusing an enucleated oocyte with blasts containing fluids other than ooplasm.

20 Ooplastoids - Ooplastoids result from the partitioning of an oocyte or ooplast. Ooplastoids are enucleated, plasma-membrane enclosed, zona pellucida free portions of the oocyte.

25 Ooplastoid/Somatic Cell Couplet -- the ooplastoid/somatic cell couplet refers to the aggregated individual somatic cell with an individual ooplast in a 1:1 ratio and prior to fusion to form the Nascent Cell.

Prophase 1 Immature Oocytes -- refers to the stage of development known as prophase 1 stage of meiosis or typically referred to as GV or germinal vesicle stage oocytes.

30 Pluripotent -- refers to cells that have the potential to develop into cells derived from all three embryonic germ layers (mesoderm, endoderm and ectoderm) of animals/humans but which do not have the ability to form into a complete human being/animal.

PNES or PNES Cells -- pluripotent non-embryonic/non-fetal tissue derived stem cells that are pluripotent and can proliferate in culture indefinitely and in an undifferentiated state.

P-PNES or P-PNES Cells – precursors to PNES that are nascent cells.

Progenitor or Precursor Cells -- immature cells that can differentiate into a limited number of different cells of the same tissue type, for example a lymphoid progenitor cell can differentiate into any one of the following: T-cells, B-cells or natural killer cells.

5        SCID Mouse -- a mouse or mouse strain with severe combined immunodeficiency (SCID) that displays profound defects in both humoral and cellular immunity.

Somatic Cells -- cells of the body carrying a diploid set of chromosomes. In humans, somatic cells carry 46 chromosomes.

Specific Differentiated Cells -- are cells derived as a result of directing PNES or ES to become multipotent/adult stem cells, and then further directing those multipotent/adult stem cells to differentiate into Specific Differentiated Cells found in animals and humans that do not have the ability to further differentiate. Examples include sertoli cells, endothelial cells, granulosa epithelial, neurons, pancreatic islet cells, epidermal cells, epithelial cells, hepatocytes, hair follicle cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, lymphocytes (B and T lymphocytes), erythrocytes, macrophages, monocytes, mononuclear cells, fibroblasts, cardiac muscle cells, and other muscle cells, etc.

Stem Cells -- all forms of stem cells have two characteristics that separate them from other cells. First, they are able to divide and replace themselves for indefinite periods. Second, at the same time that stem cells are replacing themselves they can produce cells capable of differentiating into other more specialized cells

Stem Cell Markers -- stem cell markers are cell surface molecules, usually glycoproteins, which are characteristic of a particular type of stem cell. Different stem cell lineages express unique arrays or patterns of markers that are detected using monoclonal antibodies which specifically recognize and bind to the markers.

25        Totipotent Cells -- cells that have the ability to develop into cells derived from all three embryonic germ layers (mesoderm, endoderm and ectoderm) and an entire organism (e.g., human being if placed in a woman's uterus in the case of humans). Totipotent cells may give rise to an embryo, the extra embryonic membranes and all post-embryonic tissues and organs.

Undifferentiated -- an undifferentiated cell is also an unspecialized cell that retains the potential for differentiating into other more specialized cells

Zona Pellucida Free - refers to an oocyte, oocytoïd, ooplast, or an ooplastoid from which the zona pellucida has been removed.

As used herein and in the appended claims, the singular forms "a," "an," and "the," include plural referents unless the context clearly indicates otherwise. Thus, for example,

reference to "a cell" includes one or more of such cells or a cell line derived from such a cell, "a reagent" includes one or more of such different reagents, reference to "an antibody" includes one or more of such different antibodies, and reference to "the method" includes reference to equivalent steps and methods known to those of ordinary skill in the art that could be modified or substituted for the methods described herein.

### **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 demonstrates micromanipulation of the mature mouse metaphase II oocyte: A) the mouse oocyte is oriented on the micromanipulators, B) the polar body and underlying cytoplasm containing the nuclear DNA is removed, C) formation of the enucleated oocyte is achieved by partitioning, D) shows how one mouse oocyte may be partitioned into three enucleated ooplastoids (bottom right arrow), the zona pellucida (center arrow) which is discarded, and the polar body and nuclear DNA (top center arrow) which are discarded. Bar = 100  $\mu$ m

Figure 2 shows micromanipulation and electrofusion of the ooplastoid/somatic cell couplet: A) demonstrates introduction of the somatic cell to the enucleated ooplastoid, B) shows establishing firm membrane-to-membrane contact between the ooplastoid/somatic cell couplet by pressing the somatic cell against the ooplastoid, C) shows one ooplastoid/somatic cell couplet prior to electrofusion, D) shows one ooplastoid/somatic cell couplet positioned between the electrodes in an electroporation chamber. Bar=100  $\mu$ m

Figure 3 shows the results of mitotic cell division of nascent cells at 72 h post nuclear transfer for both bovine and murine systems: A) a bovine nascent cell formed by electrofusion of an ooplastoid and a somatic cell has mitotically divided to form approximately 12 P-PNES cells, and B) a mouse nascent cell formed by the injection of a somatic cell into an ooplastoid has mitotically divided to form 8 P-PNES. Bar=100  $\mu$ m.

### **DETAILED DESCRIPTION OF THE INVENTION**

#### **Pluripotent Non-Embryonic, Non-Fetal Tissue Stem Cells (PNES)**

The present invention provides a new source for obtaining pluripotent stem cells and stem cell lines. This invention does not require the use, creation and/or destruction of embryos



or fetal tissue and does not in any way involve creating a cloned human or animal or the mixing of materials or cells between/among species. The products of this invention are pluripotent non-embryonic, non-fetal derived stem cells (PNES) and stem cell lines.

To create PNES cells, portions of the oocyte cytoplasm ("ooplastoids") are produced and combined with nuclear material of individual somatic cells. Subsequently, the newly formed P-PNES/nascent cells are cultured and give rise to PNES cells and PNES cell colonies. The oocytes and/or ooplastoids utilized in this procedure never become fertilized and never develop into embryos.

More specifically, this invention provides (a) methods of creating and culturing P-PNES cells to yield purified PNES cells which have the ability to differentiate into cells derived from mesoderm, endoderm, and ectoderm germ layers, (b) methods for maintaining and proliferating PNES cells in culture in an undifferentiated state for greater than one year, and (c) the use of those PNES cells for scientific and therapeutic purposes. These applications include, but are not limited to, use of PNES cells in (a) scientific discovery and research involving cellular development and genetic research, (b) drug development and discovery (e.g., screening for efficacy and toxicity of certain drug candidates and chemicals), (c) gene therapy (e.g., as a delivery device for gene therapy), and (d) tissue/cellular regeneration and replacement therapies and applications (e.g., replacement of damaged or destroyed blood cells, cardiac muscle, neural cells destroyed by Parkinson's, liver cells, etc.). Set forth in the remainder of this section is a detailed description of the steps and inventions described in the prior sentences.

It is important to note that this invention provides a method for deriving P-PNES cells and PNES cell lines involving unique techniques and methods, including the nuclear transfer of genetic material from a somatic cell into an enucleated, plasma membrane enclosed, zona pellucida free human ooplastoid having from 10% to 100% of the volume of ooplasm of the original egg. For description of previously reported nuclear transfer techniques, refer Campbell et al, Theriogenology, 43:181 (1995); Collas et al, Mol. Report Dev., 38:264-267 (1994); Keefer et al, Biol. Reprod., 50:935-939 (1994); Sims et al, Proc. Natl. Acad. Sci., USA, 90:6143-6147 (1993); WO 94/26884; WO 94/24274, and WO 90/03432, which are incorporated by reference in their entirety herein. Also, U.S. Pat. Nos. 4,944,384; 4,664,097; and 5,057,420 describe procedures for nuclear transplantation.). The present invention for nuclear transfer differs from those previously published in the literature in several significant ways. First, the inventor hereof was the first to announce the use of a technique wherein the zona pellucida of the oocyte used in the invention is avoided in the process of somatic cell nuclear transfer to create PNES cells. (M.J. Levanduski, Nuclear Transfer Procedure for the Production of Human Stem Cell Cultures Without Creating Embryos, 2001 International Workshop on Human and Therapeutic Cloning,

March 9, 2001 (In Press)). Subsequent to the cited report of the inventor, two other reports have been published which indicate that others are working with similar zona pellucida free techniques. A critical distinction is that the present invention involves a zona pellucida free somatic cell nuclear transfer technique that does not attempt to create an embryo. The reports cited below involve a zona pellucida free nuclear transfer technique in which the objective is to create a cloned embryo. See Simplification of Bovine Somatic Cell Nuclear Transfer by Application of Zona-Free Manipulation Technique (2001), P.J. Booth, S.J. Tan, R. Reipurth, P. Holm, H. Callesen, Cloning and Stem Cells, Vol. 3:3, 139-150; Somatic Cell Cloning Without Micromanipulators, G. Vajta, I.M. Lewis, P. Hyttel, G.A. Thouas, and A.O. Trounson (2001), Cloning, Vol. 3:2, 89-95.) Second, the present invention provides that after enucleation, the oocyte is subdivided into up to 6 membrane intact ooplastoids, having anywhere from about 10% to about 100% of the total volume of the original oocyte. Previous nuclear transfer procedures directed to creating viable cloned embryos generally utilized enucleated recipient ooplasts consisting of from about 50% to about 100% of the oocytes original volume in order to maximize ooplasm/somatic cell v/v ratio. Third, the conditions of intracytoplasmic nucleus injection, electroporation, and cell fusion (somatic cell to ooplastoid) in the present invention varies significantly compared to standard fusion techniques. In the current invention, the basic unit, ooplastoid/somatic cell aggregate, is not enclosed by a zona pellucida and therefore is very fragile and is subject to damage very easily. Fusion of the ooplastoid/somatic cell aggregate using a standard fusion chamber is described in the present invention. Accordingly, the present invention also discloses a unique fusion technique involving moveable electrodes that are introduced directly into the micromanipulation Petri dish where the ooplastoid/somatic cell aggregate is assembled and immediately electroporated to induce fusion. The present invention provides for optimized fusion and activation parameters and the resulting nascent cells (P-PNES) for all species. Finally, the techniques utilized for directing mitotically dividing P-PNES cells to become PNES cells in *in vitro* culture is herein unique

Finally, the techniques utilized for directing mitotically dividing P-PNES cells to become PNES cells in *in vitro* culture is herein unique. The inventor first reported this technique in 2001 (Procedure for the Production of Human Stem Cell Cultures Without Creating Embryos, M.Levanduski, 2001 International Workshop on Human and Therapeutic Cloning, March 9, 2001. in press). A similar technique was recently reported, however this technique in bovine involved culture of pooled embryo blastomeres to create bovine ES cells (Pluripotency of Bovine Embryonic Cell Line

Derived from Precompacting Embryos. M. Mitalipova, Z. Beyhan, and N.L. First, 2001, Cloning, vol 3, no. 2, pages 59-68.)

Source, maturation and preparation of oocytes

5        There are several actual or potential sources for human oocytes for this invention and the application thereof. First, immature human oocytes are obtained from established human *in vitro* fertilization centers with appropriate patient knowledge and consent. (The oocytes obtained via this channel are immature eggs that would otherwise be discarded. Generally human IVF patients produce approximately 10-12 oocytes per cycle, approximately 80% of which are  
10        mature metaphase oocytes capable of becoming fertilized and forming an embryo for the patient. The remaining oocytes (approximately 20%) are immature (prophase I or metaphase I) oocytes. Immature human oocytes are not capable of fertilization or creating an IVF embryo at that point and are therefore typically discarded as medical waste by the IVF laboratory).

15        A second source for human oocytes may be via a dedicated oocyte donor who donates her oocytes for a specific application for a friend or relative (e.g., a sister of a patient with a degenerative disease). A third source would be obtaining of oocytes via purchase from willing donors in conformity with all applicable laws and regulations.

20        Immature (prophase I and metaphase I) donated oocytes undergo a maturation period in specialized medium until the oocytes attain the metaphase II stage. This period of time beginning with aspiration of the immature oocyte from the ovarian follicles and including the time spent maturing the oocytes in a maturation medium and lasting until the oocyte attains the metaphase II stage is known as the maturation period. Only human oocytes which mature *in vitro* to the metaphase II stage within 36 h of oocyte retrieval are utilized further in the current invention.

25        The maturation period of the oocytes will depend on the initial stage of development of the oocyte and end stage of development desired for use. Accordingly, the oocytes are incubated for a fixed time maturation period, which ranges from about 10 to 48 h. Alternatively, the oocytes can be matured for any period of time: an oocyte can be matured for greater than 10 h, matured for greater than about 20 h, matured for greater than about 24 h, matured for greater  
30        than about 36 h, more preferably matured for greater than 48 h, even more preferably matured for greater than about 53 h, preferably matured for greater than about 60 h, preferably matured for greater than about 72 h, or matured for greater than about 90 h. The term "about" with respect to oocyte maturation can refer to plus or minus 3 h.

The present invention provides non-embryonic stem cells and methods of making them from a starting material comprising human or non-human animal oocytes. In a preferred embodiment of the present invention the source of oocyte is a human female. In certain embodiments of the present invention, the non-human animal species providing oocytes is bovine. In other embodiments, the non-human animal species providing oocytes is ovine. In still other embodiments, the non-human animal species providing oocytes is porcine. In yet other embodiments, the non-human animal species providing oocytes is caprine. Other non-human animals contemplated for providing oocytes for use in the present invention include, but are not limited to, horses (equine), dogs (canine), cats (feline), buffaloes, llamas, ferret, guinea pig, rabbits and other commercial and domestic species.

Animal oocytes were and will be secured from reputable commercial suppliers. Maturation of the oocytes followed a known standard procedure. For example, immature oocytes may be washed in HEPES buffered embryo culture medium (HECM) as described in Seshagine et al., Biol. Reprod., 40, 544-606, 1989, and then placed into drops of maturation medium consisting of tissue culture medium (TCM) 199 containing 10% fetal calf serum which contains appropriate gonadotropins such as luteinizing hormone (LH) and follicle stimulating hormone (FSH), and estradiol under a layer of lightweight paraffin or silicon at 39 C.

An alternative source for murine oocytes is via collection from mice stimulated by exogenous hormones. Mouse oocytes were obtained by inducing superovulation of 4-8 week old females (B6CBA/F1, Jackson Lab) by first administering intraperitoneal (IP) injections of 5 IU Pregnant Mare Serum Gonadotropin, (Calbiochem 367222) followed by 5 IU of hCG (Sigma). Next, the mice were sacrificed at 22 h post hCG injection and the ovaries and fallopian tubes were dissected to remove oocytes. The recovered oocytes were then washed in HECM (Conception Technologies, EH500) supplemented with 10% Plasmanate (Bayer, Elkhart, IN). Granulosa cells were removed from the oocyte preparation by treatment of 0.5-1.0 mg/ml hyaluronidase (Sigma 40K8927) followed by mechanical pipetting of the cells using a fine bore Pasteur pipette. The denuded oocytes were washed in HECM prior to micromanipulation to remove hyaluronidase residue. Only mature Metaphase II oocytes were utilized further in this procedure.

30

After maturation, but prior to enucleation, the oocytes of all species described here are denuded of surrounding granulosa cells by using a chemical treatment of HECM containing 0.5 to 1.0 mg/ml of hyaluronidase (Sigma H3757). Subsequent repeated pipetting through very fine

bore pipettes or by vortexing briefly mechanically removes the granulosa cells. The denuded oocytes are then screened for maturation status and the selected metaphase II oocytes, as determined by the presence of polar bodies, are then used for nuclear transfer. Next, the oocytes are enucleated.

5

#### Enucleation of mature metaphase II oocytes

The nucleus of the oocyte (human and animal) can be removed by standard techniques, such as described in U.S. Pat. No. 4,994,384, which is incorporated by reference herein. For example, metaphase II oocytes are placed in HECM, optionally containing 7.5-15.0 µg/ml  
10 Cytochalasin B (Sigma C6762), for immediate enucleation using micromanipulation procedures.

Enucleation may be accomplished microsurgically using a micropipette to remove the polar body and the adjacent cytoplasm after breaching the zona pellucida. The oocytes may then be screened to identify those oocytes that have been successfully enucleated. This screening may be effected by staining the oocytes with 1-5 mg/ml Hoechst 33342 dye in HECM, and then  
15 viewing the oocytes with a microscope equipped with ultraviolet irradiation for less than 10 seconds. The oocytes that have been successfully enucleated are then placed in a suitable culture medium e.g., CR2 medium (CR1 medium supplemented with amino acids), the latter of which is described in U.S. Pat. No. 5,096,822, "Bovine embryo medium," Rosenkrans Jr. et al., Nov. 3, 1992, hereby incorporated herein by reference in its entirety, including all figures, tables, and  
20 drawings. One of skill in the art would understand that a variety of culture media are used depending on the species and cell type being cultured.

The zona pellucida of the mammalian oocyte may be breached and/or removed by mechanical breaching and/or chemical breaching. Mechanical breaching and/or removal of the zona pellucida is accomplished by cutting the zona with a fine glass or metallic needle or  
25 equivalent. Chemical breaching and/or removal of the zona pellucida is accomplished by treatment with Acidic Tyrodes solution, or by treatment with a wide variety of proteases such as Pronase. Localized application of the chemical may result in a zona breach (hole) whereas treatment of the entire oocyte may result in complete dissolving of the zona pellucida.

In another method of enucleation, a glass needle (micropipette) is placed into an oocyte  
30 and the nucleus is aspirated into the needle. Thereafter, the needle can be removed from the oocyte without rupturing the plasma membrane. See, U.S. Pat. No. 4,994,384; U.S. Pat. No. 5,057,420; and Willadsen, 1986, Nature 320:63-65. An enucleated oocyte is preferably prepared from a mature metaphase II oocyte that has been matured for greater than 24 h, preferably matured for greater than 36 h

35 In the present invention, the recipient oocytes are enucleated at a time ranging from about

10 h to about 48 h after the initiation of maturation, more preferably from about 10 h to about 36 h after initiation of maturation, more preferably from about 16 h to about 24 h after initiation of maturation, and most preferably about 16 to about 18 h after initiation of maturation.

5        Ooplastoid generation

The process of ooplastoid generation in the present invention is a novel technique for the following reasons. First, in a certain embodiment of the present invention enucleated oocytes are subdivided to create plasma membrane-contained ooplastoids that have a significantly smaller volume than an intact oocyte, thus allowing the creating of multiple ooplastoids from a single  
10 oocyte. In a preferred embodiment the ooplastoid has a volume of less than 50% of a whole oocyte. More particularly, the ooplastoids have a volume from about 17% to about 33% of a whole oocyte. Second, the ooplastoid is not enclosed by a zona pellucida. There are several methods of creating these reduced volume ooplastoids. Some examples include, but are not limited to, the following:

- 15        1. Enucleated oocytes are placed in HECM containing 7.5-15.0 µg/ml Cytochalasin B. Next, the enucleated oocytes are microsurgically subdivided using micropipettes and a micromanipulation apparatus (Narashige, Japan). A portion of each enucleated oocyte is aspirated and pinched off from the oocyte leaving the ooplast plasma membrane intact. The procedure is repeated until the enucleated oocyte is subdivided into 2-6 ooplastoids,  
20        with each enucleated ooplastoid containing from about 17% to about 50% of the original volume of the intact oocyte. The ooplastoid generation procedure is repeated for each enucleated oocyte. Through this process the zona pellucida is left behind as a waste product and plays no further role in the invention.
- 25        2. In some circumstances, it may be advantageous for the ooplastoids to retain as much of the volume of the original oocyte as possible, therefore only one oocyte would yield one ooplastoid and the volume would be from about 50% to about 100% of the volume of the original oocyte.
- 30        3. The zona pellucida of the nucleated or enucleated whole oocyte may be removed chemically using standard techniques such as protease, or acidic Tyrodes solution. The zona pellucida free oocytes are placed in HECM containing 7.5-15.0 µg/ml Cytochalasin B. The zona pellucida free oocyte is then subdivided using micropipettes and a micromanipulation apparatus (Narashige, Japan). A portion of each oocyte is aspirated and pinched off from the oocyte leaving the plasma membrane intact. In one  
35        embodiment of the invention, the procedure is repeated until the enucleated oocyte is subdivided into 2-6 plasma membrane contained ooplastoids. Ooplastoids are then

screened by staining with 1-5 µg/ml Hoechst 33342 dye in HECM, and then viewing the ooplastoids with a microscope equipped with ultraviolet irradiation for less than 10 seconds. Only enucleated ooplastoids are utilized further.

5 In one embodiment, each ooplastoid contains less than 100% of the original volume of the oocyte; preferably each ooplastoid contains less than about 50% of the original volume of the oocyte. Alternatively, each ooplastoid contains less than about 30% of the original volume of the oocyte. Alternatively, each ooplastoid contains less than about 20% of the original volume of the oocyte. In another embodiment, each ooplastoid contains from about 10% to about 100%  
10 of the original volume of the oocyte. Preferably, each ooplastoid contains from about 15% to about 50% of the original volume of the oocyte. More preferably, each ooplastoid contains from about 15% to about 37% of the original volume of the oocyte. Even more preferably, each ooplastoid contains from about 17% to about 33% of the original volume of the oocyte. The ooplastoids can be human or animal ooplastoids.

15

#### Source of somatic nucleus

The ooplastoids generated above will be combined through the process of nuclear transfer with chosen somatic cells. The somatic cells in the current invention are human as well as other animal species, however it is important to reiterate that the current invention involves combining  
20 somatic cells' or somatic cells nuclei with ooplastoids of the same species, i.e. human-to-human, mouse-to-mouse, bovine-to-bovine. The human or animal somatic cells may be obtained by well-known methods. The cells used for nuclear transfer may be obtained from different organs, e.g., skin, lung, pancreas, liver, stomach, intestine, heart, reproductive organs, bladder, kidney, urethra and other urinary organs, etc., generally from any organ or tissue containing live  
25 nucleated somatic or diploid germ cells. Human and animal cells useful in the present invention include, by way of example, adult stem cells, sertoli cells, endothelial cells, granulosa epithelial, neurons, pancreatic islet cells, epidermal cells, epithelial cells, hepatocytes, hair follicle cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, lymphocytes (B and T lymphocytes), erythrocytes, macrophages, monocytes, mononuclear cells, fibroblasts, cardiac  
30 muscle cells, and other muscle cells, etc. generally any live nucleated somatic or diploid germ cell. These are just examples of suitable donor cells. The somatic cells utilized in the present invention are granulosa cells of bovine, ovine, murine, or human origin.

#### Preparation of the donor/host somatic cell

The human or animal somatic cells utilized in the current invention are cultured *in vitro*  
35 prior to nuclear transfer. In the present invention prior to nuclear transfer the human and animal

somatic (granulosa) cells are cultured in ECM supplemented with standard (10%) or alternatively reduced 0.5% concentrations of FCS or Plasmanate (Bayer). It may be necessary to induce quiescence in donor cells prior to nuclear transfer, using a suitable technique known in the art. The techniques for stopping the cell cycle at various stages have been summarized in U.S. Pat. No. 5,262,409, which is herein incorporated by reference in its entirety. For example, while cycloheximide has been reported to have an inhibitory effect on mitosis (Bowen and Wilson (1955) J. Heredity 45: 3-9), it may also be employed for improved activation of mature bovine follicular oocytes when combined with electric pulse treatment (Yang *et al.* (1992) Biol. Reprod 42 (Suppl. 1): 117).

#### Combining Somatic Cell/Nucleus With The Ooplast/Ooplastoid

In a preferred embodiment of the present invention, one individual somatic cell nucleus is transferred into one ooplastoid (a 1:1 ratio) to produce a P-PNES cell which is a nascent cell. It is important to state that the current invention involves transferring a somatic cell into an ooplastoid of the same species (i.e. human somatic cell fused to human ooplastoid, murine somatic cell to murine ooplastoid, bovine somatic cell to bovine ooplastoid, etc.). Nuclear transfer techniques are utilized in the current invention include (a) direct intracytoplasmic injection of the somatic cell nucleus into the enucleated ooplastoid, and (b) electrofusion of the entire somatic cell to the enucleated ooplastoid. Both of these techniques are utilized in human and animal species for the current invention.

Direct intracytoplasmic injection of the somatic cell nucleus into the enucleated ooplast is well known in the art of nuclear transfer. This technique is disclosed in Collas and Barnes, Mol. Reprod. Dev., 38:264-267 (1994), and incorporated by reference in its entirety herein. Briefly this involves breaking the outer membrane of the somatic and injecting the nucleus directly into the enucleated ooplast. This is accomplished utilizing an injection micropipette with a diameter smaller than the diameter of the somatic cell, thereby rupturing the somatic cell plasma membrane prior to injection of the nucleus into the enucleated ooplast. The result is that the somatic cell nucleus is effectively transferred into the intact enucleated ooplast. Activation of the oocyte may occur as a result of the intracytoplasmic injection treatment, or may be intentionally effected shortly thereafter, typically less than 24 h after injection.

The present invention provides a method where individual somatic cells and ooplastoids are fused by electrofusion. Electrofusion is accomplished by providing a pulse of electricity that is sufficient to cause a transient breakdown of the plasma membrane. This breakdown of the



plasma membrane is rapid and the membrane subsequently reforms. Basically, if two adjacent membranes are induced to breakdown and upon subsequent reformation the lipid bilayers will intermingle and small channels will open between the two independent cells. As a consequence, and due to the thermodynamic instability of such a small opening, the channels will enlarge until the two cells become one. See U.S. Pat. No. 4,997,384 to Prather *et al.*, for a further discussion of this process, which is hereby incorporated by reference in its entirety. A variety of electrofusion media can be used including e.g., sucrose, mannitol, sorbitol and phosphate buffered solution.

Electrofusion in the present invention is described in which somatic cells are successfully fused to ooplasts/ooplastoids using a commercially available fusion chamber and defined electrofusion parameters and media. It should be noted however, that using a commercially available fusion chamber can result in reduced fusion efficiency due to handling of the fragile zona pellucida free ooplastoid, somatic cell, or the ooplastoid/somatic cell couplet. Despite reduced survival and fusion efficiency of this process, successful fusion and post fusion cleavage have been achieved and described herein. For example, the human or animal cell and same species ooplastoid may be fused in a 500  $\mu\text{m}$  chamber by application of an electrical pulses of 90-120 V for about 25  $\mu\text{sec}$ /pulse. After fusion, the resultant fused P-PNES/nascent cells are then placed in a suitable medium. Activation of the ooplastoid may occur as a result of the electroporation treatment, or may be intentionally effected shortly thereafter, typically less than 24 h after fusion.

The present invention also includes an alternative electrofusion technique comprising micromanipulation of the cells and electroporation without a commercially produced electrofusion chamber. Instead the ooplastoids and somatic cells are placed in a Petri dish, or equivalent culture dish, containing fusion medium. Micropipettes are introduced and each somatic cell is paired with a single ooplastoid to create an ooplastoid/somatic cell couplet. Electrodes are then immediately introduced directly into the Petri dish, and electrical pulses are administered immediately to the couplets. The distance between the electrodes, the voltage of the pulse, the duration of the pulse, and the number of pulses are factors that influence survival of the cells and fusion success. Those of skill in the art will appreciate that optimization of fusion parameters using this system will depend on the particular species being fused, the type and size of ooplastoid, and the type of donor cell.

#### Activation of Ooplastoids, P-PNES Cells and PNES Cells

After combination of the somatic cell nucleus with the enucleated ooplastoid by injection or electrofusion, activation of the resulting P-PNES/nascent cells may be required to stimulate

development. Activation is required for human, bovine, ovine, and murine ooplasts and/or P-PNES/nascent cell; however the timing and/or technique may differ between species. One method of activation known in the art involves electrical pulses and this method is sometimes sufficient for activation of cells. The ooplastoid and or P-PNES/nascent cell may have become  
5 "activated" as a result of the intracytoplasmic injection procedure or as a result of the electrofusion procedure, in which case no additional activation treatment is required. If additional activation treatment is required, electroporation treatments may be applied. For example, the human or animal P-PNES/nascent cell may be pulsed in a 500  $\mu$ m chamber by application of repeated electrical pulses of 90-120 V for about 25  $\mu$ sec/pulse.

10 Alternatively, other non-electrical means for activation are useful and are often necessary for proper activation of an ooplastoid or P-PNES/nascent cell. See, e.g., Grocholova et al., 1997, J. Exp. Zoology 277: 49-56; Schoenbeck et al., 1993, Theriogenology 40: 257-266; Prather et al., 1989, Biology of Reproduction 41: 414-418; Prather et al., 1991, Molecular Reproduction and Development 28: 405-409; Mattioli et al., 1991, Molecular Reproduction and Development 30:  
15 109-125; Terlouw et al., 1992, Theriogenology 37: 309; Prochazka et al., 1992, J. Reprod. Fert. 96: 725-734; Funahashi et al., 1993, Molecular Reproduction and Development 36: 361-367; Prather et al., Bio. Rep. Vol. 50 Sup 1: 282; Nussbaum et al., 1995, Molecular Reproduction and Development 41: 70-75; Funahashi et al., 1995, Zygote 3: 273-281; Wang et al., 1997, Biology of Reproduction 56: 1376-1382; Piedrahita et al., 1989, Biology of Reproduction 58: 1321-1329;  
20 Machaty et al., 1997, Biology of Reproduction 57: 85-91; and Machaty et al., 1995, Biology of Reproduction 52: 753-758.

Examples of components that are useful for non-electrical activation include ethanol; inositol trisphosphate ( $IP_3$ ); divalent ions (e.g., addition of  $Ca^{2+}$  and/or  $Sr^{2+}$ ); ionophores for divalent ions (e.g., the  $Ca^{2+}$  ionophore ionomycin); protein kinase inhibitors (e.g., 6-  
25 dimethylaminopurine (DMAP)); protein synthesis inhibitors (e.g., cyclohexamide); phorbol esters such as phorbol 12-myristate 13-acetate (PMA); and thapsigargin. It is also known that temperature change and mechanical techniques are also useful for non-electrical activation. The invention includes any activation techniques known in the art. See, e.g., U.S. Pat. No. 5,496,720, entitled "Parthenogenic Oocyte Activation," issued on Mar. 5, 1996, Susko-Parrish et al., and  
30 Wakayama et al., 1998, Nature 394: 369-374, each of which is incorporated herein by reference in its entirety, including all figures, tables and drawings.

When ionomycin and DMAP are utilized for non-electrical activation, ionomycin and DMAP may be introduced to cells simultaneously or in a step-wise addition, the latter being a  
35 preferred mode as described herein. Preferred concentrations of ionomycin and DMAP are 0.5

μM ionomycin to 50 μM ionomycin and 0.5 mM DMAP to 50 mM DMAP, more preferably 1 μM ionomycin to 20 μM ionomycin and 1 mM DMAP to 5 mM DMAP, and most preferably about 10 μMolar ionomycin and about 2 mM DMAP, where the term "about" can refer to plus or minus 2 μM ionomycin and 1 mM DMAP.

5

Culture conditions of PNES or P-PNES cells, and prevention of cell clumping

P-PNES/nascent cells of all species produced by somatic cell nuclear transfer described here are cultured in ECM (Quinns Advantage Cleavage Medium, Sage Biopharma, Bedminster, NJ) supplemented with 10% Plasmanate(Bayer), HSA, or FCS at 5-6% CO<sub>2</sub> at 37° C. Each P-PNES/nascent cell in this invention is cultured individually for 72-96 h. P-PNES cells are observed using an inverted Nikon Eclipse microscope with a heated (37° C) stage at 24, 48, 72, and 96 h post micromanipulation/activation. In the human, mouse, and bovine each P-PNES/nascent cell cleaves (divides mitotically) to form two to four separate cells at about 24 h post activation, four to eight separate cells at about 48 h post activation, and eight or more cells at about 72 and about 96 h. Dividing cells at 72 to 96 h post activation may begin to form plasma membrane contact between adjacent cells. To prevent formation of cell to cell membrane connections, the cells are separated by mechanical (pipetting) treatment and chemical treatment with hyaluronidase, trypsin, chymotrypsin or similar chemical treatment in calcium and magnesium free phosphate buffered saline with 10% FCS. Mechanically separated cells originating from different P-PNES/nascent cells may be pooled at about 72 to 96 h post activation. If the pooled P-PNES/nascent cells all originated from the same somatic cell donor/source then the pooled cells are presumably autologous to each other as well as the somatic cell donor/source.

25

Culture conditions of P-PNES cells for formation of PNES cells

For human, mouse, and bovine cells, 100 to 200 pooled P-PNES cells about 72-96 hour post activation are introduced to a fibroblast feeder culture system as follows. Mouse or other animal fetal fibroblasts are isolated from postcoitum fetuses. Human fibroblasts may originate from a patient or from a screened donor. Mitomycin or ultra-violet inactivated fibroblasts are cultured in monolayers at 70,000 to 90,000 cells/cm<sup>2</sup> in Nunc 35x10 mm culture dishes, in DMEM supplemented with 10% FCS, L.I.F., and S.I.T. (Sigma), with 5-6% CO<sub>2</sub> at 37° C. Disaggregated, pooled P-PNES cells about 72-96 hour post activation are introduced and spread upon the inactivated fibroblast monolayer using a sterile Pasteur pipette. Cells are observed periodically for the next 48 h and mechanically disaggregated using a Pasteur pipette if clumps of cells are observed. This is repeated until cells are observed to adhere to the feeder layer. On

35

about day 3-7 after introducing the cells to the feeder layer the cell colonies are observed for mechanical cell sorting. Cells on the monolayer are manipulated using an inverted microscope equipped with a micromanipulator and a polished 25  $\mu$ m micropipette. Alternatively, a hand drawn sterile Pasteur pipette may be used to mechanically manipulate cultured cells while the technician is viewing the cell colonies with a stereomicroscope. Cells exhibiting embryonic stem cell like morphology (i.e., flat round or irregular shape, form loose aggregates, can form embryoid bodies) are selected and physically separated from the monolayer and aspirated into the micropipette or Pasteur pipette. See United States Patent No. 6,200,806 and Thompson, J.A. *et al. Science*, 282:1145-7, 1998. See also Amit, M., Thompson, J.A. *et al. Clonally Derived Human Embryonic Stem Cell Lines Maintained Pluripotency and Proliferative Potential For Prolonged Periods of Culture. Dev. Biol.* 227, 271-278 (2000). The selected cells are then transferred (passaged) to a new inactivated fibroblast feeder layer for continued culture. As mentioned above, these cells are referred to as pluripotent non-embryonic/non-fetal tissue derived stem cells or PNES cells. PNES cells are passaged to fresh inactivated mouse fetal fibroblast monolayer cultures about every 7-10 days according to standard embryonic stem cell culture techniques. Aliquots of these PNES cells may be characterized as stem cells using the stem cell markers. For human PNES cells are SSEA-1(-).SSEA-3(+).SSEA-4(+).TRA-1-60(+).TRA-1-81(+). The cells are to be tested using immunofluorescent microscopy. The mouse monoclonal antibodies to stage-specific embryonic antigens (SSEA) 1.3 and 4 are available from Hybridoma Bank at NIH. TRA-1-60 and TRA-1-80 are available from Vector Laboratories. To certify PNES cells for the presence or absence of the indicated markers, the cells will be placed on the cover slips pre-treated with poly-lysine or containing irradiated mouse embryonic fibroblasts (3000 rad) allowed to adhere and spread and fixed with 4% formalin. Following the fixation the cells are be stained with different antibodies and the presence of the marker is identified by binding the FITC labeled rabbit anti-mouse polyclonal antibodies. As a control the embryocarcinoma (EC) cell line NTERA-2 cl. D1 (available from ATCC) will be used.

Culture of human derived, pooled 72-96 h post activation P-PNES cells may be performed in a manner identical to that described for the mouse and bovine pluripotent ES cells. This involves using mouse fetal fibroblast monolayers as described above, a disadvantage if the cells are ultimately destined for use in cell replacement clinical therapy. Alternatively, human fibroblast monolayers may be substituted. The source of the human fibroblasts used for the continuous PNES cell culture ideally will be autologous to the source of the somatic cell used for nuclear transfer.

When grown in culture, pluripotent ES cells, and therefore PNES cells, may be inhibited from differentiation by growth on inactivated fibroblast feeder layers. Methods for isolating one

or more cells from another group of cells are well known in the art. See, e.g., Culture of Animal Cells: a manual of basic techniques (3rd edition), 1994, R. I. Freshney (ed.), Wiley-Liss, Inc.; Cells: a laboratory manual (vol. 1), 1998, D. L. Spector, R. D. Goldman, L. A. Leinwand (eds.), Cold Spring Harbor Laboratory Press; and Animal Cells: culture and media, 1994, D. C. Darling, S. J. Morgan John Wiley and Sons, Ltd.

PNES cells may be maintained in cell culture using an appropriate growth medium. PNES cell growth or culture medium means any medium that supports growth of PNES cells in culture. For example, the present invention may be practiced using a variety of human PNES cell growth media prepared on a base of Dulbecco's minimal essential media (DMEM) supplemented with 15% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, or glucose and phosphate free modified human tubal fluid media (HTF) supplemented with 15% fetal calf serum, 0.2 mM glutamine, 0.5 mM taurine, and 0.01 mM each of the following amino acids; asparagine, glycine, glutamic acid, cysteine, lysine, proline, serine, histidine, and aspartic acid (McKieman et al., Molecular Reproduction and Development 42:188-199, 1995). Typically, the medium also contains commonly used tissue culture antibiotics, such as penicillin and streptomycin. An effective amount of factors are then added daily to either of these base solutions. The term "effective amount" as used herein is the amount of such described factor as to permit a beneficial effect on human PNES cell growth and viability of human PNES cells using judgment common to those of skill in the art of cell culturing and by the teachings supplied herein.

#### Cell Culture, Maintaining Undifferentiated State And Proliferation

Mouse ES cells can be maintained in a proliferative undifferentiated state *in vitro* by growing them on feeder layers of MEF cells. An alternative to culturing on feeder layers is the addition of Leukemia inhibitory factor (LIF) to the medium. See Smith, A.G. (2001), Origins and Properties of Mouse Embryonic Stem Cells, Annu. Rev. Cell. Dev. Biol.; Williams, R. L., Hilton, D.J., Pease, S., Wilson, T.A., Stewart, C.L., Gearing, D.P., Wagner, E.F., Metcalf, D., Nicola, N.A., and Gough, N.M. (1998), Myloid Leukemia Inhibitory Factor Maintains the Developmental Potential of Embryonic Stem Cells, Nature. 336, 684-687; Rathjen, P.D., Toth, S., Willis, A., Heath, J.K., and Smith, A.G. (1990) Differentiation Inhibiting Activity is Produced in Matrix-Associated and Diffusible Forms that are Generated by Alternate Promoter Usage, Cell. 62, 1105-1114; Burdon, T, Chambers, I., Stracey, C., Niwa, H., and Smith, A. (1999). Signaling Mechanisms Regulating Self-Renewal and Differentiation of Pluripotent Stem Cells. Cells Tissues Organs 165, 131-143; Smith, A.G. (2001). Embryonic stem cells. Marshak, D.R., Gardner, D.K., and Gottlieb, D. eds. (Cold Spring Harbor, New York: Cold Spring Harbor

Laboratory Press). 205-230. Those techniques and associated publications are incorporated herein as part of this invention as they are applied to PNES cells. In contrast, even large concentrations of cloned LIF have failed to prevent differentiation of primate ES cell lines in the absence of fibroblast feeder layers. Consequently, we have found that PNES cells and primate  
5 ES stem cells are more similar to human EC cells than to mouse pluripotent ES cells, in that they are dependent on the presence of fibroblasts and will not be inhibited from differentiation by LIF in the absence of fibroblasts.

As noted, it has been demonstrated that primate and human pluripotent ES cells will continue to proliferate *in vitro* in an undifferentiated state within certain culture conditions for  
10 longer than one year, and will maintain the developmental potential to contribute to all three embryonic germ layers. See United States Patent No. 6,200,806 and Thompson, J.A. et al. *Science*, 282:1145-7, 1998. See also Amit, M., Thompson, J.A. et. al. (2000). Clonally Derived Human Embryonic Stem Cell Lines Maintained Pluripotency and Proliferative Potential For Prolonged Periods of Culture. *Dev. Biol.* 227, 271-278. There are additional methods described  
15 in additional publications which allow one to grow pluripotent stem cells in culture indefinitely and in an undifferentiated state, which are also incorporated herein and used to grow PNES cells under such conditions and achieving similar results.

#### Cryopreservation of PNES Cells

20 The PNES cells of the present invention for all species may be cryopreserved. Cells, embryos, or portions of animals are routinely frozen and stored at temperatures around -196°C. Cells and embryos can be cryopreserved for an indefinite amount of time. It is known that biological materials can be cryopreserved for more than fifty years and still remain viable. For example, bovine semen that is cryopreserved for more than fifty years can be utilized to  
25 artificially inseminate a female bovine animal and result in the birth of a live offspring. There are several programmed freezing protocols that can be used for the purpose of optimization of the survival rate for each particular cell type or each species. Methods and tools for cryopreservation are well-known to those skilled in the art. See, e.g., U.S. Pat. No. 5,160,312, entitled "Cryopreservation Process for Direct Transfer of Embryos," issued to Voelkel on Nov. 3,  
30 1992.

Alternatively, the human and non-human PNES cells of the present invention may be cryopreserved using the open pulled straw vitrification method. This method is known for the use with embryos and has recently been shown to be very effective for the use with human Pluripotent ES cells. See "Effective cryopreservation of human embryonic stem cells by the  
35 open pulled straw vitrification method," B.E. Reubinoff et al., *Human Reproduction*, 16:(10)

2187-94 (2001).

The term "thawing" as used herein can refer to a process of increasing the temperature of a cryopreserved cell, embryo, or portions of animals. Methods of thawing cryopreserved materials such that they are active after a thawing process are well-known to those of ordinary skill in the art.

#### Determining Properties and Characteristics of PNES Cells and PNES Cell Lines

In order to establish that PNES are pluripotent and can proliferate in culture for an indefinite period in an undifferentiated state, we have employed methods and practices similar, and in some cases identical, to those utilized to identify, prove and/or determine the characteristics of animal and human ES and EC cells, which have also displayed the characteristics of pluripotency, undifferentiation and proliferation. Therefore, in order to understand our methods for characterizing the qualities and attributes of PNES, one must have a solid understanding of the development of ES and EC cells in both human and animal models and the different ways in which those cells characteristics and properties have been illustrated or proven.

The mouse has been a very important model for studying pluripotent ES cells and has been a good prototype for generating, identifying and studying human pluripotent ES cells, and therefore proves helpful in defining the characteristics and properties of PNES cells for the purposes of the current invention. For example, it was first demonstrated in the mouse system that pluripotent ES cells can be maintained and propagated in an undifferentiated state (which is important to characterizing PNES cells ) provided that the mouse pluripotent ES cells are grown on feeder layer of fibroblast cells (Evans et al., Id.). Recent reports indicate that ES cell lines could be grown in an undifferentiated state without feeder layers by introducing a specific molecule or condition which inhibits differentiation is provided to allow propagation without differentiation (Smith et al., Dev. Biol., 121:1-9 (1987); see also announcements by the Xu, et al. to the effect that it has proliferated ES cell lines without the use of mouse feeder layers by substituting the mouse feeder layers with a mixture of conditioning factors including Matrigel or Laminin and MEF). Because mouse pluripotent ES cells have been shown to be able to proliferate in culture and display pluripotency (see, e.g., Evans *et al.*, Nature, 29:154-156 (1981); Martin, Proc. Natl. Acad. Sci., USA, 78:7634-7638 (1981), the tests and methods used to prove those characteristics and properties are employed with respect to PNES cells. As mentioned above, human EC lines are also pluripotent. As a result of this fact, methods for proving this characteristic and others (e.g., relating to cell morphology, immortality, karyotype, and the expression of certain cell surface markers) are relevant in characterizing PNES cells as being

pluripotent in nature.

In addition to mouse pluripotent ES cells and human EC cell lines, since 1998 there have been developments in isolating and studying primate and human pluripotent ES cells. (US Patents 5,843,780; 6,200,806; 6,090,622 and Thompson, J.A. et al. *Science*, 282:1145-7, 1998; M. J. Shambloott et al. *Proc. Natl. Acad. Sci. USA*, 95:13726-13731, 1998). Since such time it has been found that primate and human pluripotent ES cells display pluripotency, can grow in culture indefinitely in an undifferentiated state, and have normal cell morphology and karyotyping. As a result, the tests applied to human and primate pluripotent ES cells in an effort to identify these characteristics are relevant under the current invention in characterizing PNES cells.

#### Stem Cell Morphology

Both mouse and primate pluripotent ES cells have the characteristic morphological features of undifferentiated stem cells, with high nuclear/cytoplasmic ratios, prominent nucleoli, and compact colony formation. PNES cells will display similar colony and cell morphology as the stem cells created/isolated and identified using prior technologies for animal and human pluripotent ES cells. For a broader description of cell morphologies of stem cells, see United States Patent No. 6,200,806 and Thompson, J.A. et al. *Science*, 282:1145-7, 1998, the texts of which are hereby incorporated by reference.

#### Cell surface markers

Cell surface markers have also been used as supplemental proofs to identify and isolate pluripotent stem cells. There are general cell surface markers used to identify stem cells for all species, and certain cell surface markers used to identify the stem cells for a specific species only. The general cell surface markers provide supplemental proof that PNES cells are in fact stem cells, and the species-specific cell surface markers provide supplemental proof that within that species PNES cells are stem cells.

*Available markers:* Human and animal pluripotent stem cells are usually characterized by expression of the family of markers comprising the stage-specific embryonic antigens 1 - 4 (SSEA 1-4), which are cell surface glycolipids that are expressed in early embryonic development and on the surface of pluripotent stem cells. Antibodies recognizing stage-specific embryonic antigens, SSEA 1, SSEA-3 and SSEA-4 are particularly useful in characterizing human and animal stem cells. See NIH Report *Stem Cells: Scientific Progress and Future Research Directions*, Appendix E Stem Cell Markers (2001), incorporated herein, and available at <http://www.nih.gov/news/stemcell/scireport.htm>). In addition, antibodies to



SSEA 1-4 are available for use in fluorescence activated cell sorting analysis. The antibodies can be obtained from the Developmental Studies Hybridoma Bank of the National Institute of Child Health and Human Development. There are other antigens associated with the extracellular matrix of pluripotent stem cells that are known as surface markers TRA-1-60 and TRA-1-81. (See "Cell Lines from Human Germ Cell Tumors," In: Robertson E, ed. Teratocarcinomas and Embryonic Stem Cells: A Practical Approach. Oxford: IRL Press, 207-246, 1987). As mentioned, the antibodies used to characterize human ES, EC cells and mouse pluripotent ES cells are also useful in characterizing the PNES cells of the present invention.

*Methods for using cell surface markers.* In order to detect the presence of stem cell antigens on the surface of the cells, the antibodies are first bound to the cells and subsequently a biotinylated secondary antibody containing an avidin-biotinylated horseradish peroxidase complex is used to detect that an antibody antigen has occurred (Vectastain ABC System, Vector Laboratories). ).

Human EC and mouse pluripotent ES cells lines provide important antibody controls for characterizing PNES cells and ES cell lines. Human EC and mouse pluripotent ES cells lines can be distinguished based on the expression of SSEA-1, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81. In general, pluripotent human EC cell lines are negative for SSEA-1, and are positive for SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81. Therefore, a human EC cell line may be used for comparison with a candidate pluripotent stem cell line. For example, the cell line NTERA-2 cl. D1, is a pluripotent human EC cell line that has been extensively studied and reported in the literature. See Andrews et al., "Cell lines from human germ cell tumors," In: Robertson E, ed. Teratocarcinomas and Embryonic Stem Cells: A Practical Approach. Oxford: IRL Press, 207-246, 1987. This cell line as well as many other available cell lines may serve as a positive control. In contrast, Mouse ES cells are positive for SSEA-1, and are for negative for SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81. Therefore these cell lines can be used as a negative control for SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81.

The surface expression of certain characteristic stem cell markers on mouse pluripotent ES cells, primate pluripotent ES cells, and human EC cells are shown in Table 1. As is evident from Table 1, primate pluripotent ES cells and human EC cells both express the combination of markers SSEA-3; SSEA-4, TRA-1-60, and TRA-1-81. The glycoproteins SSEA-3 and SSEA-4 are consistently present on human EC cells, and are of diagnostic value in distinguishing human EC cell tumors from human yolk sac carcinomas, choriocarcinomas, and other lineages which lack these markers. See Wenk *et al.*, Int J Cancer 58:108-115, 1994. A recent survey found SSEA-3 and SSEA-4 to be present on all of over 40 human EC cell lines examined (Wenk *et al.* Int J Cancer 58:108-115, 1994). The antigens known as TRA-1-60 and

TRA-1-81 have been well characterized on a particular pluripotent human EC cell line, NTERA-2 CL. D1. See "Cell lines from human germ cell tumors," In: Robertson E, ed. Teratocarcinomas and Embryonic Stem Cells: A Practical Approach. Oxford: IRL Press, 207-246, 1987.

Interestingly, once NTERA-2 CL. D1 cells begin to differentiate *in vitro* expression of SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 is lost, while expression of the glycoprotein SSEA-1 is increased. In contrast, undifferentiated mouse pluripotent ES cells express SSEA-1, and do not express SSEA-3 or SSEA-4. A successful PNES cells cell culture prepared according to the present invention will be consistent with the patterns of cell surface markers described in Table 1.

Table 1 shows that human EC cells and human pluripotent ES cells are indistinguishable based on expression of the described markers. Therefore, these two types of cells may be distinguished on the basis of karyotype. As described above, human and primate pluripotent ES cells maintain a normal euploid karyotype while human EC cells are typically aneuploid and thus easily distinguished.

**Table 1 Marker Expression of ES and EC Cell Lines**

Marker	Stem Cell Lines		
	Human EC	Mouse ES	Human ES
SSEA-1	Negative	Positive	Negative
SSEA-3	Positive	Negative	Positive
SSEA-4	Positive	Negative	Positive
TRA-1-60	Positive	Negative	Positive
TRA-1-81	Positive	Negative	Positive

There are several cell surface markers which are used to indicate the characteristics of pluripotent PNES under the current invention including, but not limited to, those found on Table 2.

**Table 2**

PLURIPOTENT STEM CELLS		
Marker Name	Cell Type	Significance
Alkaline phosphatase	Embryonic stem (ES), embryonal carcinoma (EC)	Elevated expression of this enzyme is associated with undifferentiated pluripotent stem cell (PSC)

Alpha-fetoprotein (AFP)	Endoderm	Protein expressed during development of primitive endoderm; reflects endodermal differentiation
Bone morphogenetic protein-4	Mesoderm	Growth and differentiation factor expressed during early mesoderm formation and differentiation
Brachyury	Mesoderm	Transcription factor important in the earliest phases of mesoderm formation and differentiation; used as the earliest indicator of mesoderm formation
Cluster designation 30 (CD30)	ES, EC	Surface receptor molecule found specifically on PSC
Crypto (TDGF-1)	ES, cardiomyocyte	Gene for growth factor expressed by ES cells, primitive ectoderm, and developing cardiomyocyte
GATA-4 gene	Endoderm	Expression increases as ES differentiates into endoderm
GCTM-2	ES, EC	Antibody to a specific extracellular-matrix molecule that is synthesized by undifferentiated PSCs
Genesis	ES, EC	Transcription factor uniquely expressed by ES cells either in or during the undifferentiated state of PSCs
Germ cell nuclear factor	ES, EC	Transcription factor expressed by PSCs
Hepatocyte nuclear factor-4 (HNF-4)	Endoderm	Transcription factor expressed early in endoderm formation
Nestin	Ectoderm, neural and pancreatic progenitor	Intermediate filaments within cells; characteristic of primitive neuroectoderm formation
Neuronal cell-adhesion molecule (N-CAM)	Ectoderm	Cell-surface molecule that promotes cell-cell interaction; indicates primitive neuroectoderm formation
Oct-4	ES, EC	Transcription factor unique to PSCs; essential for establishment and maintenance of undifferentiated PSCs
Pax6	Ectoderm	Transcription factor expressed as ES cell differentiates into neuroepithelium
Stage-specific embryonic antigen-3 (SSEA-3)	ES, EC	Glycoprotein specifically expressed in early embryonic development and by undifferentiated PSCs
Stage-specific embryonic antigen-4 (SSEA-4)	ES, EC	Glycoprotein specifically expressed in early embryonic development and by undifferentiated PSCs
Stem cell factor (SCF or c-kit ligand)	ES, EC, HSC, MSC	Membrane protein that enhances proliferation of ES and EC cells, hematopoietic stem cell (HSCs), and mesenchymal stem cells (MSCs); binds the receptor c-kit
Telomerase	ES, EC	An enzyme uniquely associated with immortal cell lines; useful for identifying undifferentiated PSCs
TRA-1-60	ES, EC	Antibody to a specific extracellular matrix molecule is synthesized by undifferentiated PSCs
TRA-1-81	ES, EC	Antibody to a specific extracellular matrix molecule normally synthesized by undifferentiated PSCs

Vimentin	Ectoderm, neural and pancreatic progenitor	Intermediate filaments within cells; characteristic of primitive neuroectoderm formation
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*Application of cell markers to PNES cells.* The PNES cells of the present invention are positive for alkaline phosphatase, similar to the situation found with pluripotent ES cells. For example, pluripotent ES cells all are known to express alkaline phosphatase and monitoring this enzyme can be useful during the isolation, culturing and characterization of these cells. The expression of alkaline phosphatase is shared by both primate and mouse pluripotent ES cells, and relatively few other embryonic cells express this enzyme. Positive cells include the ICM and primitive ectoderm (which are the most similar embryonic cells in the intact embryo to pluripotent ES cells), germ cells (which are totipotent), and a very limited number of neural precursors. See Kaufman M H. The atlas of mouse development. London: Academic Press, 1992.

#### Pluripotency

Pluripotency has been proven by injecting candidate ES cells into mice with severe combined immunodeficiency (SCID) and analyzing the cell types comprising the resulting tumors, which have been shown to differentiate into cells representing all three germ layers. All selected PNES cell lines are injected into mice with SCID and are able to differentiate into cells representing all three germ layers. For example, approximately  $0.5-1.0 \times 10^6$  candidate PNES cells are injected into the rear leg muscles or testis of 8-12 week old male SCID mice (6-10 mice) and let grow until forming the tumor-like cell mass. The resulting tumors are fixed in 4% paraformaldehyde and examined histologically after paraffin embedding at 8-16 weeks of development. Next, the embedded tumors are sectioned and cell types comprising the tumor are evaluated. In the preferred embodiment, PNES cells demonstrate the ability to differentiate into the following: cartilage, smooth muscle, and striated muscle (mesoderm); stratified squamous epithelium with hair follicles, neural tube with ventricular, intermediate, and mantle layers (ectoderm); ciliated columnar epithelium and villi lined by absorptive enterocytes and mucus-secreting goblet cells (endoderm). It should be noted that these are only a few of the cell types that may be present in the tumors and this list is not meant to be exhaustive.

Multiple techniques for proving pluripotency for mouse ES cells re described in Smith A.G. (2001), Origins and Properties of Mouse Embryonic Stem Cells, Annu. Rev. Cell. Dev. Biol., which such report and techniques/methods are incorporated herein and is used under the current invention to prove pluripotency. These methods include methods similar to that described above, and also a technique under which the feeder layers are removed and leukemia

inhibitory factor (LIF) is added to the growth medium, and within a few days of changing the culture conditions, pluripotent cells (PNES cells or ES cells) aggregate and may form embryoid bodies (EB) which consist of cells which are both differentiated and partially differentiated that are derived from the three primary germ layers.

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### Karyotype

The present invention provides human and animal PNES cells that have normal karyotypes, similar to what has been seen in other stem cells (human and nonhuman ES lines). In addition, both XX and XY cells lines will be derived. A normal karyotype indicates that all  
10 chromosomes normally characteristic of the species are present and have not been noticeably altered. Cell lines can be karyotyped with a standard G-banding technique (such as by the Cytogenetics Laboratory of the University of Wisconsin State Hygiene Laboratory, which provides routine karyotyping services) and compared to published karyotypes for the primate species.

15 A karyotype is the particular chromosome complement of an individual or of a related group of individuals, as defined both by the number and morphology of the chromosomes usually in mitotic metaphase. It includes such things as total chromosome number, copy number of individual chromosome types (e.g., the number of copies of chromosome X), and chromosomal morphology, e.g., as measured by length, centromeric index, connectedness, or the  
20 like. Chromosomal abnormalities can be detected by examination of karyotypes. Karyotypes are conventionally determined by staining a cell's metaphase, or otherwise condensed (for example, by premature chromosome condensation) chromosomes.

A number of cytological techniques based upon chemical stains have been developed which produce longitudinal patterns on condensed chromosomes, generally referred to as bands.  
25 The banding pattern of each chromosome within an organism usually permits unambiguous identification of each chromosome type, Latt, "Optical Studies of Metaphase Chromosome Organization," Annual Review of Biophysics and Bioengineering Vol. 5, pgs. 1-37 (1976). Accurate detection of some important chromosomal abnormalities, such as translocations and inversions, has required such banding analysis.

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### Immortality

The PNES cells of the present invention are immortal. Immortal cells are capable of continuous indefinite replication *in vitro*. As a practical matter, immortality is measured by observing continued proliferation of cells for longer than one year in culture. Likewise, primary  
35 cell cultures that are not immortal fail to continuously divide for this length of time. See

Freshney, Culture of animal cells. New York: Wiley-Liss, 1994. It has been demonstrated that primate and human pluripotent ES cells will continue to proliferate *in vitro* with the culture conditions described below for longer than one year, and will maintain the developmental potential to contribute to all three embryonic germ layers. See United States Patent No.

5 6,200,806 and Thompson, J.A. et al. *Science*, 282:1145-7, 1998. The methods described and utilized by Thompson are incorporated herein by reference as one of the methods deployed under the current invention to grow PNES *in vitro* for an indefinite period and in an undifferentiated state. Note that to date, it has not been demonstrated that the pluripotent stem cells generated from embryonic germ cells have this property. U.S. Patent 6,090,622 and M. J. Shambloott *et al.*  
 10 *Proc. Natl. Acad. Sci. USA*, 95:13726-13731, 1998.

Whether a candidate PNES cell line has retained the proper developmental potential along with its immortality can be determined by injecting the PNES cell lines into SCID mice after being grown and maintained in culture for one year. In the preferred embodiment, the PNES cell lines are cultured for the time period in question, usually 1 year, and then about 0.5-  
 15  $1.0 \times 10^6$  candidate PNES cells are injected into the rear leg muscles or testis of 8-12 week old male SCID mice (6-10 mice). The resulting tumors can be fixed in 4% paraformaldehyde and examined histologically after paraffin embedding at 8-16 weeks of development. It is possible that karyotypic changes can occur randomly in some cells with prolonged culture, however some PNES cells will maintain a normal karyotype for longer than a year of continuous culture as  
 20 proven by the tests for karyotyping described above.

### **Multipotent/Adult Stem Cells (ASC's) and Specific Differentiated Cells**

#### *Directing Differentiation of Pluripotent PNES to ASC's and Specific Differentiated Cells.*

There are various and differing techniques and methods for directing PNES cells to become  
 25 different types of ASC's and Specific Differentiated Cells *in vitro*, including, but not limited to, into the following cell types: adipocyte, astrocyte, cardiomyocyte, chondrocyte, definitive hematopoietic, dendritic, endothelial, keratinocyte, lymphoid precursor, mast, neuron, oligodendrocyte, osteoblast, pancreatic islets, primitive hematopoietic, smooth muscle, striated muscle, yolk sac endoderm, and yolk sac mesoderm. As evidenced, these techniques can be  
 30 utilized to direct pluripotent human cells such as PNES into cells derived from all three germ layers, and publications describing those techniques cited here and the relevant techniques described therein are incorporated completely under the current invention and are used to prove similar results with respect to PNES and derivatives thereof. Kehar, I., Kenyagin-Karsenti, D., Druckmann, M., Segev, H., Amit, M., Gepstein, A., Livne, E., Binah, O., Itskovitz-Eldor, J., and  
 35 Gepstein, L. (2001). Human ES cells can differentiate into myocytes portraying cardiomyocytic

structural and functional properties. J. Clin. Invest. (In press); Itskovitz-Eldor, J., Schuldiner, M., Karsenti, D., Eden, A., Yanuka, O., Amit, M., Soreq, H., and Benvenisty, N. (2000). Differentiation of human embryonic stem cells into embryoid bodies comprising the three embryonic germ layers. Mol. Med. 6, 88-95; Assady, S., Maor, G., Amit, M., Itskovitz-Eldor, J., Skorecki, K.L., and Tzukerman, M. (2001). Insulin production by human embryonic stem cells. Diabetes, 50; and Kerr, D.A., Llado, J., Shambloot, M., Maragakis, N., Irani, D.N., Dike, S., Sappington, A., Gearhart, J., and Rothstein, J. (2001). Human embryonic germ cell derivatives facilitate motor recovery of rats with diffuse motor neuron injury.

Some additional specific examples include methods for directing pluripotent human stem cells into bone, cartilage, squamous and cuboidal epithelium, neural cells, glandular epithelium and striated muscle, and the techniques relating to directing PNES cells into those particular types of cells as described in the following citations are also incorporated completely under the current invention and are used to prove similar results with respect to PNES cells and derivatives thereof. See Reubinoff, B.E., Pera, M.F., Fong, C.Y., Trounson, A., and Bongso, A. (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation *in vitro*. Nat. Biotechnol. 18, 399-404; and Roach, S., Cooper, S., Bennett, W., and Pera, M.F. (1993). Cultured cell lines from human teratomas: windows into tumor growth and differentiation and early human development. Eur. Uro. 23, 82-87. In general terms, to aid in understanding the underlying techniques themselves, the methods for directing pluripotent stem cells to become ASC's and Specific Differentiated Cells include, but are not limited to, (a) adding growth factors to the culture medium or changing the chemical composition of the surface on which the pluripotent cells are growing, and (b) introducing foreign genes into the pluripotent cells via transfection or other methods, the result of which is to add an active gene to the pluripotent cell genome which then triggers the cells to differentiate along a particular pathway, c) co-culturing with inactivated primary specialized cells or tissues, or in the presence of those tissue matrix components, d) using media supplemented with the extracts prepared from the specialized tissues and/or organs.

The techniques and methods of differentiation described in the following publications and the publications cited therein are herein incorporated by reference in their entirety under the current invention and are used to provide similar results with respect to PNES cells and derivatives thereof.

#### **ADIPOCYTE**

Dani, C., Smith, A.G., Dessolin, S., Leroy, P., Staccini, L., Villageois, P., Darimont, C., and Ailhaud, G. (1997). Differentiation of embryonic stem cells into adipocytes *in vitro*. J. Cell. Sci. 110, 1279-1285.

**ATROCYTE**

Fraichard, A., chassandre, O., bilbaut, G., Dehay, C., Savatier, P., and Samarut, J. (1995). In vitro differentiation of embryonic stem cells into glial cells and functional neurons.

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**CARDIOMYOCYTE**

Doetschman, T., Eistetter, H., Katz, M., Schmit, w., and Kemler, R. (1985). The in vitro development of blastocysts-derived embryonic stem cell lines: formatoin of visceral yolk sac, blood islands and myocardium. J. Embryol. Exp. Morph. 87, 27-45.

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Maltsev, V.A., rohwedel, J., Hescheler, J., and Wobus, A.M. (1993). Embryonic stem cells differentiate in vitro into cardiomyocytes representing sinusnodal, atrial and ventricular cell types. Mech. Dev. 44, 41-50.

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**CHONDROCYTE**

Kramer, J., Hegert, C., Guan, K., Wobus, A.M., Muller, P.K., and Rohwedel, J. (2000). Embryonic stem cell-derived chondrogenic differentiation in vitro: activation by BMP-2 and BMP-4. Mech. Dev. 92, 193-205.

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**DEFINITIVE HEMATOPOIETIC**

Nakano, T., Kodama, H., and Honjo, T. (1996). In vitro development of primitive and definitive erythrocytes from different precursors. Science. 272, 722-724.

Nishikawa, S., Hirashima, M., Matsuyoshi, N., and Kodama, H. (1998). Progressive lineage analysis by cell sorting and culture identifies FLK1(+)VE-cadherin(+) cells at a diverging point of endothelial and hemopoietic lineages. Development. 125, 1747-1757.

Wiles, M.V. and Keller, G. (1991). Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture. Development. 111, 259-267.

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**DENDRITIC CELL**

Fairvhild, P.J., Brook, F.A., Gardner, R.L., Graca, L., Strong, V., Tone, Y., Tone, M., Nolan, K.F., and Waldmann, H. (2000). Directed differentiation of dendritic cells from mouse embryonic stem cells. Curr. Biol. 10, 1515-1518.

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**ENDOTHELIAL CELL**

Risau, W., Sariolola, H., Zerwes, H.G., Sasse, J., Ekblom, P., Kemler, R., and Doetschman, T. (1988). Vasculogenesis and angiogenesis in embryhonic-stem-cell-derived embryoid bodies. Development. 102, 471-478.

Yamashita, J., Itoh, H., Hirashima, M., Ogawa, M., Nishikawa, S., Yurugi, T., Naito, M., Nakao, K., and Nishikawa, S. (2000). Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. Nature. 408, 92-96.

**KERATINOCYTE**

Bagutti, C., Wobus, A.M., Fassler, r., and Watt, f.M. (1996). Differentiation of

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embryonal stem cells into keratinocytes: comparison of wild-type and B(1) integrin-deficient cells. *Dev. Biol.* 179, 184-196.

- 5 Yamashita, J., Itoh, H., Hirashima, M., Ogawa, M., Nishikawa, S., Yurugi, T., Naito, M., Nakao, K., and Nishikawa, S. (2000). Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. *Nature.* 408, 92-96.

#### **LYMPHOID PRECURSOR**

- 10 Potocnik, A.J., Nielsen, P.J., and Eichmann, K. (1994). In vitro generation of lymphoid precursors from embryonic stem cells. *EMBO. J.* 13, 5274-5283.

#### **MAST CELL**

- 15 Tsai, M., Wedemeyer, J., Ganiatsas, S., Tam, S.Y., Zon, L.I., and Galli, S.J. (2000). In vivo immunological function of mast cells derived from embryonic stem cells: an approach for the rapid analysis of even embryonic lethal mutations in adult mice in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 97, 9186-9190.

#### **NEURON**

- 20 Bain, G., Kitchens, d., Yao, M., Huettner, J.E., and Gottlieb, D.I. (1995). Embryonic stem cells express neuronal properties in vitro. *Dev. Biol.* 168, 342-357.

- 25 Strubing, C., Ahnert-Hilger, G., Shan, J., Wiedenmann, B., Hescheler, J., and Wobus, A.M. (1995). Differentiation of pluripotent embryonic stem cells into the neuronal lineage in vitro gives rise to mature inhibitory and excitatory neurons. *Mech. Dev.* 53, 275-287.

#### **OLIGODENDROCYTE**

- 30 Brustle, O., Jones, K.N., Learish, R.D., Karram, K., Choudhary, K., Wiestler, O.D., Duncan, I.D., and McKay, R.D. (1999). Embryonic stem cell-derived glial precursors: a source of myelinating transplants. *Science.* 285, 754-756.

- 35 Liu, S., Qu, Y., Stewart, T.J., Howard, M.J., Chakraborty, S., Holekamp, T.F., and McDonald, J.W. (2000). Embryonic stem cells differentiate into oligodendrocytes and myelinate in culture and after spinal cord transplantation. *Proc. Natl. Acad. Sci. U.S.A.* 97, 6126-6131.

#### **OSTEOBLAST**

- 40 Buttery, L.D., Bourne, S., Xynos, J.D., Wood, H., Hughes, F.J., Hughes, S.P., Episkopou, V., and Polak, J.M. (2001). Differentiation of osteoblasts and in vitro bone formation from murine embryonic stem cells. *Tissue Eng.* 7, 89-99.

#### **PANCREATIC ISLETS**

- 45 Lumelsky, N., Biondel, O., Laeng, P., Velasco, I., Ravin, R., and McKay, R. (2001). Differentiation of Embryonic Stem Cells to Insulin-Secreting Structures Similar to Pancreatic Islets. *Science.* 292, 1389-1394.

#### **PRIMITIVE HEMATOPOIETIC**

Doetschman, T., Eistetter, H., Katz, M., Schmit, w., and Kemler, R. (1985). The in vitro development of blastocysts-derived embryonic stem cell lines: formatoin of visceral yolk sac, blood islands and myocardium. J. Embryol. Exp. Morph. 87, 27-45.

- 5 Nakano, T., Kodama, H., and Honjo, T. (1996). In vitro development of primitive and definitive erythrocytes from different precursors. Science. 272, 722-724.

#### **SMOOTH MUSCLE**

- 10 Yamashita, J., Itoh, H., Hirashima, M., Ogawa, M., Nishikawa, S., Yurugi, T., Naito, M., Nakao, K., and Nishikawa, S. (2000). Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. Nature. 408, 92-96.

#### **STIRATED MUSCLE**

- 15 Rohwedel, J., Maltsev, V., Bober, e., Arnold, J.J., Hescheler, J., and Wobus, A.M. (1994). Muscle cell differentiation of embryonic stem cells reflects myogenesis in vivo: developmentally regulated expression of myogenic determination genes and functional expression of ionic currents. Dev. Biol. 164, 87-101.

#### **YOLK SAC ENDODERM**

- 20 Doetschman, T., Eistetter, H., Katz, M., Schmit, w., and Kemler, R. (1985). The in vitro development of blastocysts-derived embryonic stem cell lines: formatoin of visceral yolk sac, blood islands and myocardium. J. Embryol. Exp. Morph. 87, 27-45.

#### **YOLK SAC MESODERM**

- 25 Doetschman, T., Eistetter, H., Katz, M., Schmit, w., and Kemler, R. (1985). The in vitro development of blastocysts-derived embryonic stem cell lines: formatoin of visceral yolk sac, blood islands and myocardium. J. Embryol. Exp. Morph. 87, 27-45.

- 30 *Cell surface markers.* There are various cell surface markers employed under the current invention to isolate, identify and define the characteristics of the ASC's and/or Specific Differentiated Cells created under the current invention including, but not limited to, those described on Table 3 which are incorporated herein.

**TABLE 3**

<b>MARKERS COMMONLY USED TO IDENTIFY ADULT STEM CELLS AND TO CHARACTERIZE DIFFERENTIATED OR SPECIFIC CELL TYPES</b>		
<b>Marker Name</b>	<b>Cell Type</b>	<b>Significance</b>
<b>BLOOD VESSELL</b>		
Fetal liver kinase-1 (Flk1)	Endothelial	Cell-surface receptor protein that identifies endothelial cell progenitor; marker of cell-cell contacts

Smooth muscle cell-specific myosin heavy chain	Smooth muscle	Identifies smooth muscle cells in the wall of blood vessels
Vascular endothelial cell cadherin	Smooth muscle	Identifies smooth muscle cells in the wall of blood vessels
<b>BONE</b>		
Bone-specific alkaline phosphatase (BAP)	Osteoblast	Enzyme expressed in osteoblast; activity indicates bone formation
Hydroxyapatite	Osteoblast	Mineralized bone matrix that provides structural integrity; marker of bone formation
Osteocalcin (OC)	Osteoblast	Mineral-binding protein uniquely synthesized by osteoblast; marker of bone formation
<b>BONE MARROW AND BLOOD</b>		
Bone morphogenetic protein receptor (BMPR)	Mesenchymal stem and progenitor cells	Important for the differentiation of committed mesenchymal cell types from the mesenchymal stem and progenitor cells; BMPR identifies early mesenchymal lineages (stem and progenitor cells)
CD4 and CD8	White blood cell (WBC)	Cell-surface protein markers specific for mature T lymphocyte (WBC subtype)
CD34	Hematopoietic stem cell (HSC), satellite, endothelial progenitor	Cell-surface protein on bone marrow cell, indicative of a HSC and endothelial progenitor; CD34 also identifies muscle satellite, a muscle stem cell
CD34+Scal+Lin-profile	Mesenchymal stem cell (MSC)	Identifies MSCs, which can differentiate into adipocyte, osteocyte, chondrocyte, and myocyte
CD38	Absent on HSC Present on WBC lineages	Cell-surface molecule that identifies WBC lineages. Selection of CD34+/CD38- cells allows for purification of HSC populations
CD44	Mesenchymal	A type of cell-adhesion molecule used to identify specific types of mesenchymal cells
c-Kit	HSC, MSC	Cell-surface receptor on BM cell types that identifies HSC and MSC; binding by fetal calf serum (FCS) enhances proliferation of ES cells, HSCs, MSCs, and hematopoietic progenitor cells
Colony-forming unit (CFU)	HSC, MSC progenitor	CFU assay detects the ability of a single stem cell or progenitor cell to give rise to one or more cell lineages, such as red blood cell (RBC) and/or white blood cell (WBC) lineages
Fibroblast colony-forming unit (CFU-F)	Bone marrow fibroblast	An individual bone marrow cell that has given rise to a colony of multipotent fibroblastic cells; such identified cells are precursors of differentiated mesenchymal lineages

Hoechst dye	Absent on HSC	Fluorescent dye that bind DNA; HSC extrudes the dye and stains lightly compared with other cell types
Leukocyte common antigen (CD45)	WBC	Cell-surface protein on WBC progenitor
Lineage surface antigen (Lin)	HSC, MSC  Differentiated RBC and WBC lineages	Thirteen to 14 different cell-surface proteins that are markers of mature blood cell lineages; detection of Lin-negative cells assists in the purification of HSC and hematopoietic progenitor populations
Mac-1	WBC	Cell-surface protein specific for mature granulocyte and macrophage (WBC subtypes)
Muc-18 (CD146)	Bone marrow fibroblasts, endothelial	Cell-protein (immunoglobulin superfamily) found on bone marrow fibroblasts, which may be important in hematopoiesis; a subpopulation of Muc-18+ cells are mesenchymal precursors
Stem cell antigen (Sca-1)	HSC, MSC	Cell-surface protein on bone marrow (BM) cell, indicative of HSC and MSC
Stro-1 antigen	Stromal (mesenchymal) precursor cells, hematopoietic cells	Cell-surface glycoprotein on subsets of bone marrow stromal (mesenchymal) cells; selection of Stro-1+ cells assists in isolating mesenchymal precursor cells, which are multipotent cells that give rise to adipocyte, osteocyte, smooth myocyte, fibroblasts, chondrocyte, and blood cells
Thy-1	HSC, MSC	Cell-surface protein; negative or low detection is suggestive of HSC
<b>CARTILAGE</b>		
Collagen types II IV	Chondrocyte	Structural proteins produced specifically by chondrocyte
Keratin	Keratinocyte	Principal protein of skin; identifies differentiated keratinocyte
Sulfated proteoglycan	Chondrocyte	Molecule found in connective tissues; synthesized by chondrocyte
<b>FAT</b>		
Adipocyte lipid-binding protein (ALBP)	Adipocyte	Lipid-binding protein located specifically in adipocyte
Fatty acid transporter (FAT)	Adipocyte	Transport molecule located specifically in adipocyte

Adipocyte lipid-binding protein (ALBP)	Adipocyte	Lipid-binding protein located specifically in adipocyte
<b>GENERAL</b>		
Y chromosome	Male cells	Male-specific chromosome used in labeling and detecting donor cells in female transplant recipients
Karyotype	Most cell types	Analysis of chromosome structure and number in a cell
<b>LIVER</b>		
Albumin	Hepatocyte	Principal protein produced by the liver; indicates functioning of maturing and fully differentiated hepatocytes
B-1 integrin	Hepatocyte	Cell-adhesion molecule important in cell-cell interactions; marker expressed during development of liver
<b>NERVOUS SYSTEM</b>		
CD133	Neural stem cell, HSC	Cell-surface protein that identifies neural stem cells, which give rise to neurons and glial cells
Glial fibrillary acidic protein (GFAP)	Astrocyte	Protein specifically produced by astrocyte
Microtubule-associated protein-2 (MAP-2)	Neuron	Dendrite-specific MAP; protein found specifically in dendritic branching of neuron
Myelin basic protein (MPB)	Oligodendrocyte	Protein produced by mature oligodendrocytes; located in the myelin sheath surrounding neuronal structures
Nestin	Neural progenitor	Intermediate filament structural protein expressed in primitive neural tissue
Neural tubulin	Neuron	Important structural protein for neuron; identifies differentiated neuron
Neurofilament (NF)	Neuron	Important structural protein for neuron; identifies differentiated neuron
Neurosphere	Embryoid body (EB), ES'	Cluster of primitive neural cells in culture of differentiating ES cells; indicates presence of early neurons and glia
Noggin	Neuron	A neuron-specific gene expressed during the development of neurons
O4	Oligodendrocyte	Cell-surface marker on immature, developing oligodendrocyte
O1	Oligodendrocyte	Cell-surface marker that characterizes mature oligodendrocyte

Synaptophysin	Neuron	Neuronal protein located in synapses; indicates connections between neurons
Tau	Neuron	Type of MAP; helps maintain structure of the axon
<b>PANCREAS</b>		
Cytokeratin 19 (CK19)	Pancreatic epithelium	CK19 identifies specific pancreatic epithelial cells that are progenitors for islet cells and ductal cells
Glycogen	Pancreatic islet	Expressed by alpha-islet cell of pancreas
Insulin	Pancreatic islet	Expressed by beta-islet cell of pancreas
Insulin-promoting factor-1 (PAX-1)	Pancreatic islet	Transcription factor expressed by beta-islet cell of pancreas
Nestin	Pancreatic progenitor	Structural filament protein indicative of progenitor cell lines including pancreatic
Pancreatic polypeptide	Pancreatic islet	Expressed by gamma-islet cell of pancreas
Somatostatin	Pancreatic islet	Expressed by delta-islet cell of pancreas
<b>SKELETAL MUSCLE/CARDIAC/SMOOTH MUSCLE</b>		
MyoD and Pax7	Myoblast, Myocyte	Transcription factors that direct differentiation of myoblasts into mature myocytes
Myogenin and MR4	Skeletal myocyte	Secondary transcription factors required for differentiation of myoblasts from muscle stem cells
Myosin heavy chain	Cardiomyocyte	A component of structural and contractile protein found in cardiomyocyte
Myosin light chain	Skeletal myocyte	A component structural and contractile protein found in skeletal myocyte

**Summary – Isolation and Differentiation Of PNES Cells , ASC's and Specific Differentiated Cells**

As indicated throughout this detailed discussion, there are many techniques and methods for isolating, identifying, differentiation and directing PNES cells, ASC's and Specific Differentiated Cells. Many of these techniques are summarized in the following references, which are hereby incorporated by reference in their entirety methods of performing these tasks under the current invention.

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20 **Applications Of PNES Cells And Advantages Of PNES Cells**  
**Relative To Other Pluripotent Cell Lines**

Scientific and therapeutic applications of the technology and composition of this invention include, but are not limited to, the following:

1. Studies on human development and the origin of the disease. Help understand  
25 complexities of formation of human organs and tissues. Most major diseases are due to abnormal cell specialization and cell division. PNES cells give us a key research tool for understanding fundamental events in human development, such as explaining the causes of birth defects, and approaches to prevent or correct.
2. Drug discovery, drug evaluation, drug testing and drug development. To test a drug or  
30 chemical's efficacy or toxicity, the scientific community currently uses animal models *in vitro* using cells from rats, mice and other animals, or *in vivo* tests that involve giving the drug or chemical to the animal to test safety. Beside the ethical considerations, these tests/models are not always predictive for what will happen in human beings. Human models to date usually involve established cell lines that have been maintained *in vitro*  
35 for a long period of time. These cell lines are usually transformed and differ significantly from primary cells *in vivo*, making these established cell lines of limited utility. PNES cells can help in overcoming many if not all of these shortcomings.
3. Treatment of diseases and disorders including, but not limited to, Parkinson's, Alzheimer's, Huntington's, Ty Sachs, Gauchers, spinal cord injury, stroke, burns and



other skin damage, heart disease, diabetes, Lupus, osteoarthritis, liver diseases, hormone disorders, kidney disease, leukemia, lymphoma, multiple sclerosis, rheumatoid arthritis, Duchenne's Musclar Dystrophy, Ontogenesis Imperfecto, birth defects, infertility, pregnancy loss, and other cancers, degenerative and other diseases and disorders.

- 5       4. Genomics/Gene Manipulation/Delivery Devices. Scientists predict that human stem cells such as PNES will be useful vehicles for delivering genes to specific tissues. The current alternative, viral delivery devices, have significant limitations (e.g., some viruses only attack dividing cells, not all cells, so application is limited, and there are risks of harmful immune reaction associated with this mechanism). PNES cells can offer a more robust
- 10       delivery system that can overcome these limitations.

In addition to providing these promising applications, PNES cells also have characteristics and properties that make them a more attractive alternative when compared with ES cell lines created under current technologies. These advantages include, but are not limited to, the following.

- 15       1. The creation of PNES cells doesn't involve embryos (naturally created or created via cloning), fetal tissue or the mixing of species.
2. Current ES cell lines come from a limited genetic pool whereas PNES cell lines can be created from an unlimited genetic pool and can be created specifically for a given patient or patient population (e.g., PNES can be autologous) and thus PNES cells avoid another
- 20       likely barrier to the use of ES cell lines - - immune rejection.
3. PNES cell lines can be created on an ongoing basis, whereas because of certain limitations imposed by the NIH and proposed legislation, the creation of new ES cell lines for human is under severe scrutiny and faces significant barriers. The ES cell lines that currently exist and are approved for federally funded applications will likely be
- 25       subject to genetic changes and mutations as they age, e.g., they can't be kept healthy in culture indefinitely.
4. PNES cells for humans can be created and proliferated in cultures without using mouse feeding layers, so as to avoid the mixing of species.

30       **Applications of Invention's ASC's and Specific Differentiated Cells and Advantages Over Other Sources:**

The application of the ASC's and Specific Differentiated Cells created by the current invention include, but not exclusively,

- 35       1. Drug discovery, drug evaluation, drug testing and drug development. To test a drug or chemical's efficacy or toxicity, the scientific community currently uses animal models in

vitro using cells from rats, mice and other animals, or in vivo tests that involve giving the drug or chemical to the animal to test safety. Beside the ethical considerations, these tests/models are not always predictive for what will happen in human beings. Human models to date usually involve established cell lines that have been maintained in vitro for a long period of time. These cell lines are usually transformed and differ significantly from primary cells *in vivo*, making these established cell lines of limited utility. ASC's and Specific Differentiated Cells, which the current invention can produce on an ongoing basis including multiple cell lines, can help in overcoming many if not all of these shortcomings.

2. Treatment of diseases and disorders including, but not limited to, Parkinson's, Alzheimer's, Huntington's, Ty Sachs, Gauchers, spinal cord injury, stroke, burns and other skin damage, heart disease, diabetes, Lupus, osteoarthritis, liver diseases, hormone disorders, kidney disease, leukemia, lymphoma, multiple sclerosis, rheumatoid arthritis, Duchenne's Musclar Dystrophy, Ontogenesis Imperfecto, birth defects, infertility, pregnancy loss, and other cancers, degenerative and other diseases and disorders
3. Genomics/Gene Manipulation/Delivery Devices. Scientists predict that human stem cells such as PNES will be useful vehicles for delivering genes to specific tissues. The current alternative, viral delivery devices, have significant limitations (e.g., some viruses only attack dividing cells, not all cells, so application is limited, and there are risks of harmful immune reaction associated with this mechanism). PNES cells can offer a more robust delivery system that can overcome these limitations.

In addition to providing these promising applications, ASC's and Specific Differentiated Cells produced under the current invention have characteristics and properties that make them a more attractive alternative when compared with multipotent/adult stem cells produced or secured from other sources (such as in vivo, umbilical cords and other limited sources):

1. Under the current invention, ASC's and Specific Differentiated Cells can be produced without the use and destruction of embryos (naturally created or created via cloning), fetal tissue or the mixing of species.
2. Current methods for producing multipotent/adult stem cells and Specific Differentiated Cells (derived from current ES lines) utilize a limited genetic pool whereas multipotent ASC's and Specific Differentiated Cells produced under the current invention can be created from an unlimited genetic pool and can be created specifically for a given patient or patient population (e.g., cells produced under this invention can be autologous) and thus this invention's source for these cells avoids another likely barrier to the use of these

cells derived from ES cell lines - - immune rejection.

3. This invention can create multipotent ASC's and Specific Differentiated Cells on an ongoing basis, whereas because of certain limitations imposed by the NIH and proposed legislation, the creation of new ES cell lines for humans and derivatives thereof including multipotent and undifferentiated cells is under severe scrutiny and faces significant barriers, and the current ES cell lines and derivatives thereof will likely be subject to problems as they age such as genetic changes and mutations - - e.g., they can't be kept healthy in culture indefinitely.
4. ASC's from in vivo sources have not been identified for all human tissues whereas PNES have the ability to differentiate into cells derived from all three embryonic germ layers.
5. In vivo sourced ASC's and Specific Differentiated Cells are in short supply and costly to accumulate or harvest. The current invention offers a more efficient and productive source.
6. In vivo ASC's are much more difficult to isolate than ASC's created under the current invention.

### **DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS**

In order that this invention may be better understood, the following examples are set forth. These examples are for the purpose of illustration only and are not to be construed as limiting the scope of the invention in any manner.

5

#### **EXAMPLE I**

##### **Maturation of Bovine Oocytes**

Bovine metaphase II oocytes were obtained from a commercial source (Ovagenix, San Angelo, TX). The supplier obtained immature oocytes from a slaughterhouse source. Immature oocytes were washed in HEPES buffered embryo culture medium (HECM supplemented with 10% FCS). Next, the supplier placed immature oocytes into maturation medium consisting of tissue culture medium (TCM) 199 containing 10% fetal calf serum which contains appropriate gonadotropins such as luteinizing hormone (LH) and follicle stimulating hormone (FSH), and estradiol. The commercial supplier then placed the maturing oocytes in a battery powered portable incubator, and shipped the incubator via overnight mail to arrive in our laboratory the next morning. Therefore the maturation period occurred while the oocytes were in transit. The maturation period is defined as period beginning from the time of introducing the immature oocytes into the maturation medium until the time at which the mature oocytes are utilized in the present study. The current invention utilizes bovine mature metaphase II oocytes with a 18 to 36 hour maturation period. Mature metaphase II bovine oocytes were washed in HECM. Unwanted granulosa cells were removed from the oocytes by treatment consisting of incubating the cells in a solution of 0.5-1.0 mg/ml hyaluronidase (Sigma H3757) followed by mechanical pipetting of the cells using a fine bore Pasteur pipette. Next, the denuded oocytes were washed in HECM prior to micromanipulation to remove any hyaluronidase residue. Only mature Metaphase II bovine oocytes of normal quality were utilized further in this procedure.

#### **EXAMPLE II**

##### **Micromanipulation and Enucleation of Bovine Oocytes**

Micromanipulation and enucleation of bovine oocytes was performed as follows. Micromanipulation was performed on a inverted microscope (Nikon, Japan) using micromanipulators (Narashige, Japan). The mature metaphase II oocytes were introduced to HECM containing 10% Plasmanate and 7.5-15.0 µg/ml cytochalasin B (Sigma C6762). Next, a holding micropipette (Humagen, Charlottesville, VA) was used to grasp the oocytes. While

holding the oocyte, the zona pellucida of each oocyte was partially dissected (dissolved) by application of an acidic tyrodes solution (Sigma T1788). The acidic tyrodes solution was applied using a 20-30  $\mu$ m diameter micropipette (Humagen, Charlottesville, VA). The zona was dissolved adjacent to the polar body of the mature oocyte. Following breach of the zona, a 20-50  $\mu$ m micrometer polished micropipette (Humagen, Charlottesville, VA) was used to gently aspirate the polar body and underlying cytoplasm, which was pinched away from the remaining ooplasm. This procedure was repeated for each oocyte. The resulting "enucleated" oocytes and the removed polar body and underlying ooplasm were stained using 5  $\mu$ g/ml Hoechst 33342 (Sigma) and microscopically viewed briefly (<10 seconds) using ultraviolet irradiation to confirm that all nuclear DNA has been removed from the enucleated oocytes. Only successfully enucleated oocytes were utilized further.

### **EXAMPLE III**

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#### **Ooplastoid Generation From Bovine Oocytes**

Ooplastoid generation for bovine oocytes was performed as follows. Enucleated oocytes were introduced to HECM containing 10% Plasmanate and 7.5-15.0  $\mu$ g/ml cytochalasin B. A micromanipulator (Narashige, Japan) was used to manipulate the enucleated oocytes. A holding micropipette (Humagen 10MPH-120, Charlottesville, VAAA) was used to grasp and orient the enucleated oocytes. A 20-50  $\mu$ m polished micropipette (Humagen custom, Charlottesville, VA) was used to gently aspirate and pinch off a portion of the enucleated oocyte. This process was repeated until each enucleated oocyte was partitioned into 3-5 zona pellucida free ooplastoids having from 20 to 33% of the volume of the original oocyte. This procedure was repeated until each enucleated oocyte was appropriately partitioned into ooplastoids. Ooplastoids were washed in HECM with 10% Plasmanate to remove Cytochalasin B for further micromanipulation.

### **EXAMPLE IV**

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#### **Preparation of Bovine Somatic Cells for Nuclear Transfer**

The source of bovine somatic cell nucleus for experiments described here has been granulosa cells. Granulosa cells were obtained from bovine oocyte/granulosa masses. The granulosa masses were subjected to chemical treatment with 0.5-1.0 mg/ml hyaluronidase (Sigma H3757) followed by mechanical removal of granulosa through repeated pipetting of the

cells using fine bore Pasteur pipettes. Subsequently, the isolated granulosa cells were washed with HECM with 10% Plasmanate to remove hyaluronidase. Next, granulosa were cultured in ECM or HECM supplemented with 10% FCS or Plasmanate in preparation for further micromanipulation. Alternatively, granulosa or any other type of somatic cell may be cultured in  
5 ECM supplemented with 0.5% fetal calf serum or Plasmanate for 24 to 72 h to induce quiescence prior to nuclear transfer.

### **EXAMPLE V**

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#### **Nuclear Transfer of Somatic Cell Nuclei to Bovine Ooplastoids using Electrofusion and Creation of Nascent Cells/P-PNES**

Nuclear transfer of bovine somatic cell nuclei to ooplastoids was performed by cell electrofusion. For bovine ooplastoids electrofusion was performed as follows.  
15 Micromanipulation of ooplastoids and granulosa was performed using a micromanipulator (Narashige, Japan). A 10-20  $\mu$ m polished micropipette was used to aspirate a single granulosa cell. The granulosa cell was positioned firmly against the plasma membrane of a single ooplastoid, using mechanical pressure to maximize cell-to-cell contact. During this step the HECM may be supplemented with 100-200  $\mu$ g/ml Phytohaemagglutinin to improve cell-to-cell  
20 contact. This procedure was repeated for each ooplastoid resulting in the formation of ooplastoid/somatic cell aggregates or pairs.

The ooplastoid/somatic cell aggregates were then very gently aspirated and moved to a fusion chamber (BTX) containing fusion medium (0.3 M mannitol, 0.1mM  $\text{MgSO}_4$ , 0.05mM  $\text{CaCl}_2$ ). Next, using an electroporator, model (BTX 2001) two DC pulses of 0.1-2.0 kilovolts/cm  
25 and 25  $\mu$ s were applied to the fusion chamber to induce cell fusion. After electroporation the ooplastoid/somatic cell aggregates were gently removed from the fusion chamber and incubated in ECM with 20% Plasmanate or FCS. Cell fusion was visually confirmed or denied 20-30 minutes post electroporation by observation using an inverted microscope (Nikon, Japan). Successfully fused pairs were referred to as P-PNES or "nascent cells." The P-PNES were  
30 moved to a 30 mm Petri dish (Nunc, Denmark) containing culture medium (Quinns Advantage Cleavage Medium, Sage Biopharma, Bedminster, NJ) supplemented with 10% Plasmanate or FCS and cultured in 6%  $\text{CO}_2$ . P-PNES were observed for cleavage division over the next 72 h.

35

### **EXAMPLE VI**

#### **Activation of Bovine Ooplastoids, or P-PNES Cells**

Activation of bovine oocytes, ooplastoids, or nascent cells is a specific procedure that  
5 may be applied at one or more times during the overall laboratory process described here.  
Activation may be mechanical (simply pricking the cell with a fine bore needle or injection  
pipette), electrical (applying a DC pulse as in electrofusion), or chemical (calcium ionophore or  
ethanol treatment). Activation may be applied to the mature oocyte prior to the  
micromanipulation procedures. Depending on the species and conditions, activation may be  
10 achieved during enucleation of the oocyte, ooplastoid partitioning, or during intracytoplasmic  
injection of the somatic cell nucleus. Activation may also be achieved during the application of  
the DC pulse during the electrofusion process. In the current invention bovine P-PNES cells  
were activated as a result of electrofusion DC pulse with acceptable levels of activation achieved  
in each case. Alternatively, the frequency of successful activation may be enhanced by adding a  
15 pre or post micromanipulation activation step if improvements are desired for this critical  
process.

### **EXAMPLE VII**

#### **Superovulation and Collection of Mouse Oocytes**

20 Murine (mouse) oocytes were obtained by inducing superovulation of 4-8 week old  
females (B6CBA/F1, Jackson Lab) by first administering intraperitoneal (IP) injections of 5 IU  
Pregnant Mare Serum Gonadotropin, (Calbiochem 367222) and 5 IU of hCG (Sigma). Next, the  
25 mice were sacrificed at 22 h post hCG injection and the ovaries and fallopian tubes were  
dissected to remove oocytes. The recovered oocytes were then washed in HECM (Conception  
Technologies, EH500) supplemented with 10% Plasmanate (Bayer, Elkhart, IN). Granulosa  
cells were removed from the oocyte preparation by treatment of 0.5-1.0 mg/ml hyaluronidase  
(Sigma H3757) followed by mechanical pipetting of the cells using a fine bore Pasteur pipette.  
30 The denuded oocytes were washed in HECM prior to micromanipulation to remove  
hyaluronidase residue. Only mature metaphase II mouse oocytes were utilized further in this  
procedure.

35

### **EXAMPLE VIII**

#### **Micromanipulation and Enucleation of Mouse Oocytes**

Micromanipulation and enucleation of mouse oocytes was performed as follows.

5 Micromanipulation was performed on a inverted microscope (Nikon, Japan) using micromanipulators (Narashige, Japan). The MII Mature oocytes were introduced to HECM containing 10% Plasmanate and 7.5-15.0 µg/l cytochalasin B (Sigma C6762). Next, a holding micropipette (Humagen, Charlottesville, VA) was used to grasp the oocytes (Figure 1A). While holding the oocyte, the zona pellucida of each oocyte was partially dissected (dissolved) by  
10 application of an acidic tyrodes solution (Sigma T1788). The acidic tyrodes solution was applied using a 20-30µm diameter micropipette (Humagen, Charlottesville, VA). The zona was dissolved adjacent to the polar body of the mature oocyte. Following breach of the zona a 20-50 µm micrometer polished micropipette (Humagen, Charlottesville, VA) was used to gently aspirate the polar body and underlying cytoplasm, which was pinched away from the remaining  
15 ooplasm (Figure 1B). This procedure was repeated for each oocyte. The resulting “enucleated” oocytes and the removed polar body and underlying ooplasm was stained using 5 µg/ml Hoechst 33342 (Sigma) and viewed briefly (<10 seconds) using ultraviolet irradiation to confirm that all nuclear DNA has been removed from the enucleated oocytes. Only successfully enucleated oocytes were utilized further.

20

### **EXAMPLE IX**

#### **Ooplastoid Generation From Mouse Oocytes**

25 Ooplastoid generation for mouse oocytes was performed as follows. Enucleated oocytes were introduced to HECM containing 10% Plasmanate and 7.5-15.0 µg/ml Cytochalasin B. A micromanipulator (Narashige, Japan) was used to manipulate the enucleated oocytes. A holding micropipette (Humagen 10MPH-120, Charlottesville, VA) was used to grasp and orient the enucleated oocytes. A 20-50 µm polished micropipette (Humagen custom, Charlottesville, VA)  
30 was used to gently aspirate and pinch off a portion of the enucleated oocyte (Figure 1C). This process was repeated until each enucleated oocyte was partitioned into 2-6 zona pellucida-free ooplastoids having from about 17% to less than 50% of the volume of the original oocyte (Figure 1D). This procedure was repeated until each enucleated oocyte was appropriately partitioned into ooplastoids. Ooplastoids were washed in HECM with 10% Plasmanate to remove  
35 Cytochalasin B for further micromanipulation.



## **EXAMPLE X**

### **Preparation of Mouse Somatic Cells for Nuclear Transfer**

The source of mouse somatic cell nucleus for experiments described here has been  
5 granulosa cells. Granulosa cells were obtained from mouse oocyte/granulosa masses. The  
granulosa masses were subjected to chemical treatment with 0.5-1.0 mg/ml hyaluronidase  
(Sigma H3757) followed by mechanical removal of granulosa through repeated pipetting of the  
cells using fine bore Pasteur pipettes. Subsequently, the isolated granulosa cells were washed  
with HECM with 10% Plasmanate to remove hyaluronidase. Next, granulosa were cultured in  
10 ECM or HECM supplemented with 10% Plasmanate in preparation for further  
micromanipulation. Alternatively, granulosa or any other type of somatic cell may be cultured in  
ECM supplemented with 0.5% fetal calf serum or Plasmanate for 24 to 72 h to induce quiescence  
prior to nuclear transfer.

15

## **EXAMPLE XI**

### **Nuclear Transfer of Somatic Cell Nucleus by Direct Intracytoplasmic Injection**

Nuclear transfer of mouse somatic cell nucleus to the ooplastoids may be achieved by cell  
20 fusion or by direct intracytoplasmic injection. Direct injection of granulosa nuclei into mouse  
ooplastoids was performed as follows. Micromanipulation of ooplastoids and granulosa was  
performed using a micromanipulator (Narashige, Japan). A blunt or pointed injection  
micropipette with a 8-15  $\mu\text{m}$  diameter, slightly smaller than the granulosa cell, was used to pick  
up one granulosa cell. The granulosa cell was repeatedly aspirated and expelled from the pipette  
25 in order to break the cell membrane. The granulosa cell was immediately injected into a single  
ooplastoid, which was gently grasped by a holding pipette. The medium used for this  
micromanipulation was HECM with 10% Plasmanate and may be supplemented with 7.5-15.0  
 $\mu\text{g/ml}$  Cytochalasin B to minimize cell lysis. This procedure was repeated for each ooplastoid.  
Each successfully injected ooplastoid containing a single granulosa cell nucleus is referred to as  
30 a P-PNES. The P-PNES were moved to a 30 mm Petri dish (Nunc, Denmark) containing culture  
medium (Quinns Advantage Cleavage Medium, Sage Biopharma, Bedminster, NJ) supplemented  
with 10% Plasmanate or FCS and cultured in 6% CO<sub>2</sub>. P-PNES were observed for cleavage  
division over about the next 72-96 h.

35

## **EXAMPLE XII**

### **Activation of Mouse Oocytes, Ooplastoids, and P-PNES cells**

Activation of oocytes, ooplastoids or P-PNES cells is a specific procedure that may be applied at one or more times during the overall laboratory process described here. Activation may be mechanical (simply pricking the cell with a fine bore needle or injection pipette), electrical (applying a DC pulse as in electrofusion), or chemical (calcium ionophore or ethanol treatment). Activation may be applied to the mature oocyte prior to the micromanipulation procedures. Depending on the species and conditions, activation may be achieved during enucleation of the oocyte, ooplastoid partitioning, or during intracytoplasmic injection of the somatic cell nucleus. Activation may also be achieved during the application of the DC pulse during the electrofusion process. In the current invention a portion of the mouse ooplastoids or P-PNES cells were activated as a result of intracytoplasmic nucleus injection. The frequency of successful activation of mouse ooplastoids P-PNES cells was enhanced by adding a post micromanipulation activation step consisting of electroporation. This involved moving the P-PNES cells to a fusion chamber (BTX) containing fusion medium (0.3 M mannitol, 0.1mM MgSO<sub>4</sub>, 0.05mM CaCl<sub>2</sub>). Next, using an electroporator, model (BTX 2001) two DC pulses of 0.1-2.0 kv/cm and 25μs were applied to the electroporation.

20

## **EXAMPLE XIII**

### **Culture of Human, Mouse, and Bovine PNES or P-PNES Cells and Prevention of Cell Clumping**

P-PNES/nascent cells of all species produced by somatic cell nuclear transfer are cultured in ECM (Quinns Advantage Cleavage Medium, Sage Biopharma, Bedminster, NJ) supplemented with 10% Plasmanate(Bayer), HSA, or FCS at 5-6% CO<sub>2</sub> at 37° C. Each P-PNES/nascent cell in this invention is cultured individually for about 72 to about 96 h. P-PNES cells are observed using an inverted Nikon Eclipse microscope with a heated (37° C) stage at about 24, 48, 72, and 96 h post micromanipulation/activation. In the human, mouse, and bovine each P-PNES/nascent cell will cleave (divide mitotically) to form about two to four separate cells at about 24 h post activation, fabout our to eight separate cells at about 48 h post activation, and about eight or more cells at about 72 to 96 h. Dividing cells at about 72 to 96 h post activation begin to form plasma membrane contact between adjacent cells. To prevent formation of cell to cell membrane connections, the cells are separated by mechanical (pipetting) treatment and chemical treatment

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with hyaluronidase, trypsin, chymotrypsin or similar chemical treatment in calcium and magnesium free phosphate buffered saline with 10% FCS. Mechanically separated cells originating from different P-PNES/nascent cells are pooled at about 72 to 96 h post activation. Pooled P-PNES cells all originated from the same somatic cell donor/source are presumed  
5 autologous to each other as well as the somatic cell donor/source.

#### **EXAMPLE XIV**

##### **Culture of Human, Mouse, and Bovine P-PNES Cells for Formation of PNES Cells**

For human, mouse, and bovine cells, 100 to 200 pooled P-PNES cells at about 72 to 96 h post activation are introduced to a fibroblast feeder culture system as follows. For culture human, mouse, and bovine P-PNES cells mouse fetal fibroblasts are isolated from postcoitum fetuses. Mitomycin or ultra-violet inactivated fibroblasts are cultured in monolayers at 70,000 to  
15 90,000 cells/cm<sup>2</sup> in Nunc 35x10 mm culture dishes, in DMEM supplemented with 10% FCS, L.I.F., and S.I.T. (Sigma), with 5-6% CO<sub>2</sub> at 37° C. Alternatively, for culture of human P-PNES cells at about 72 to 96 h post activation, human fibroblast monolayers may be substituted. The source of the human fibroblasts used for the continuous PNES culture ideally is autologous to the source of the somatic cell used for nuclear transfer, however screened donor fibroblast cultures  
20 may be substituted.

Disaggregated, pooled P-PNES cells at about 72 to 96 hour post activation are introduced and spread upon the inactivated fibroblast monolayer using a sterile Pasteur pipette. Cells are observed periodically for the about next 48 h and mechanically disaggregated using a Pasteur pipette if clumps of cells are observed. This is repeated until cells are observed to adhere  
25 to the feeder layer. On about day 3 to 7 after introducing the cells to the feeder layer the cell colonies are observed for mechanical cell sorting. Cells on the monolayer are manipulated using an inverted microscope equipped with a micromanipulator and a polished 25 µm micropipette. Alternatively, a hand drawn sterile Pasteur pipette is used to mechanically manipulate cultured cells while the technician is viewing the cell colonies with a stereomicroscope. Cells exhibiting  
30 embryonic stem cell like morphology as defined by Thompson (United States Patent No. 6,200,806) are selected and physically separated from the monolayer and aspirated into a micropipette or Pasteur pipette. The selected cells are then transferred (passaged) to a new inactivated fibroblast feeder layer for continued culture. As mentioned above, these cells are referred to as pluripotent non-embryonic stem cells or PNES. PNES cells are passaged to a new  
35 inactivated fibroblast monolayer culture about every 7 to 10 days according to standard

embryonic stem cell culture techniques.

### **EXAMPLE XV**

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#### **ANALYSIS OF PNES CELLS**

Aliquots of these human, mouse, and bovine PNES cells are characterized as stem cells using the stem cell markers. For human PNES cells are SSEA-1(-).SSEA-3(+).SSEA-4(+).TRA-1-60(+).TRA-1-81(+). The cells are tested using immunofluorescent microscopy. The mouse monoclonal antibodies to stage-specific embryonic antigens (SSEA) 1.3 and 4 are available from Hybridoma Bank at NIH. TRA-1-60 and TRA-1-80 are available from Vector Laboratories. To certify PNES cells for the presence or absence of the indicated markers, the cells are placed on the cover slips on an irradiated mouse embryonic fibroblasts (3000 rad) allowed them to adhere and spread, and fixed with 4% formalin. Following fixation and staining with different antibodies the presence of the marker is identified by binding the FITC labeled rabbit anti-mouse polyclonal antibodies. As a control the embryocarcinoma (EC) cell line NTERA-2 cl. D1 (available from ATCC) are used.

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### **EXAMPLE XVI**

#### **Method For Constructing Super-Ooplasts That Are Greater Than The Size Of A Normal Oocyte**

Ooplasts may theoretically be of any size or volume. In contrast to constructing ooplast that are by volume smaller than an oocyte, ooplasts may be constructed that are actually larger than a normal oocyte. To create large ooplasts, several oocytes of any mammals species are enucleated in HECM containing 10% FCS and about 7.5-15.0 µg/ml Cytochalasin B (Sigma C6762) using micromanipulation techniques as previously described. The zona pellucida of all enucleated oocytes is removed using mechanical action or using chemical agents. The enucleated oocytes (ooplasts) are then introduced into a fusion chamber containing a fusion medium such as 0.3 M mannitol, 0.1 mM MgSO<sub>4</sub>, 0.05 mM CaCl<sub>2</sub>. Within the fusion chamber two or more ooplasts are aligned with membrane-to-membrane contact in an axis perpendicular to the electrodes. Using an electroporator one or more electrical pulses are applied with defined parameters such as 0.1-2.0 kilovolts/cm, 25 µs/pulse. After applying the pulse the ooplasts may fuse to form a non-nucleated super-ooplast consisting of a volume greater than one normal

oocyte. This may be repeated to form super-ooplasts of theoretically any volume.

It is contemplated that the invention includes methods of producing and utilizing PNES cells and their derivatives, i.e., Specific Differentiated Cells including, but not limited to sertoli cells, endothelial cells, granulosa epithelial, neurons, pancreatic islet cells, epidermal cells, epithelial cells, hepatocytes, hair follicle cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, lymphocytes (B and T lymphocytes), erythrocytes, macrophages, monocytes, mononuclear cells, fibroblasts, cardiac muscle cells, and other muscle cells, etc. in scientific and therapeutic applications including, but not limited to, (a) scientific discovery and research involving cellular development and genetic research, (b) drug development and discovery (e.g., screening for efficacy and toxicity of certain drug candidates and chemicals), (c) gene therapy (e.g., as a delivery device for gene therapy), and (d) treatment of diseases and disorders including, but not limited to, Parkinson's, Alzheimer's, Huntington's, Ty Sachs, Gauchers, spinal cord injury, stoke, burns and other skin damage, heart disease, diabetes, Lupus, osteoarthritis, liver diseases, hormone disorders, kidney disease, leukemia, lymphoma, multiple sclerosis, rheumatoid arthritis, Duchenne's Musclar Dystrophy, Ontogenesis Imperfecto, birth defects, infertility, pregnancy loss, and other cancers, degenerative and other diseases and disorders.

While we have hereinbefore described a number of embodiments of this invention, it is apparent that our basic constructions can be altered to provide other embodiments that utilize the processes and compositions of this invention. Therefore, it will be appreciated that the scope of this invention is to be defined by the claims appended hereto rather than by the specific embodiments that have been presented hereinbefore by way of example.

### CLAIMS

1. A purified preparation of pluripotent non-embryonic stem cells, which  
(i) is capable of proliferating in an *in vitro* culture for more than one year;  
5 (ii) maintains a karyotype in which the cells are euploid and are not altered through culture;  
(iii) maintains the potential to differentiate into cell types derived from the endoderm, mesoderm and ectoderm lineages throughout the culture, and  
(iv) is inhibited from differentiation when cultured on fibroblast feeder layers.  
10
2. The pluripotent non-embryonic stem cells of claim 1, wherein said cells are negative for expression of the SSEA-1 marker.
3. The pluripotent non-embryonic stem cells of claim 1, wherein said cells express  
15 elevated alkaline phosphatase activity.
4. The pluripotent non-embryonic stem cells of claim 1, wherein said cells are positive for expression of the TRA-1-81 marker and the TRA-1-60 marker.
- 20 5. The pluripotent non-embryonic stem cells of claim 1, wherein said cells are positive for expression of the CCA-3 and CCA-4 Markers.
6. The pluripotent non-embryonic stem cells of claim 1, wherein said cells differentiate into cells derived from mesoderm, endoderm and ectoderm germ layers when the  
25 cells are injected into a SCID mouse.
7. The pluripotent non-embryonic stem cells of claim 1, wherein said cells are human.
- 30 8. The pluripotent non-embryonic stem cells of claim 1, wherein said cells are non-human animal cells selected from the group consisting of dog, cat, mouse, rat, cow, pig, sheep, goat, horse, buffalo, llama, ferret, guinea pig and rabbit.
9. The pluripotent non-embryonic stem cells of claim 1, wherein the nuclear DNA  
35 has been genetically modified.

10. A purified preparation of pluripotent non-embryonic stem cells, which  
(i) is capable of proliferating in an *in vitro* culture for an indefinite period;  
(ii) maintains a karyotype in which the cells are euploid and are not altered  
5 through culture; and  
(iii) maintains the potential to differentiate into cells types derived from the  
endoderm, mesoderm and ectoderm lineages throughout the culture.
11. The pluripotent non-embryonic stem cells of claim 10, wherein said cells are  
10 negative for expression of the SSEA-1 marker.
12. The pluripotent non-embryonic stem cells of claim 10, wherein said cells express  
elevated alkaline phosphatase activity.
13. The pluripotent non-embryonic stem cells of claim 10, wherein said cells are  
15 positive for expression of the TRA-1-81 marker and the TRA-1-60 marker.
14. The pluripotent non-embryonic stem cells of claim 10, wherein said cells are  
positive for expression of the CCA-3 and CCA-4 Markers.  
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15. The pluripotent non-embryonic stem cells of claim 10, wherein said cells  
differentiate into cells derived from mesoderm, endoderm and ectoderm germ layers when the  
cells are injected into a SCID mouse.
16. The pluripotent non-embryonic stem cells of claim 10, wherein said cells are  
25 human.
17. The pluripotent non-embryonic stem cells of claim 10, wherein said cells are non-  
human animal cells selected from the group consisting of dog, cat, mouse, rat, cow, pig, sheep,  
30 goat, horse, buffalo, llama, ferret, guinea pig and rabbit.
18. The pluripotent non-embryonic stem cells of claim 10, wherein the nuclear DNA  
has been genetically modified.

19. A stem cell which does not originate from a fertilized egg, but which originates from the combination of a somatic cell nucleus and an enucleated ooplastoid.

5        20. The stem cells of claim 19, wherein said cells are negative for expression of the SSEA-1 marker.

10        21. The stem cells of claim 19, wherein said cells express elevated alkaline phosphatase activity.

22. The stem cells of claim 19, wherein said cells are positive for expression of the TRA-1-81 marker and the TRA-1-60 marker.

15        23. The stem cells of claim 19, wherein said cells are positive for expression of the CCA-3 and CCA-4 Markers.

24. The stem cells of claim 19, wherein said cells differentiate into cells derived from mesoderm, endoderm and ectoderm germ layers when the cells are injected into a SCID mouse.

20        25. The stem cells of claim 19, wherein said cells are human.

26. The stem cells of claim 19, wherein said cells are non-human animal cells selected from the group consisting of dog, cat, mouse, rat, cow, pig, sheep, goat, horse, buffalo, llama, ferret, guinea pig and rabbit.

25        27. The stem cells of claim 19, wherein the nuclear DNA has been genetically modified.

30        28. The stem cells of claim 19, wherein said enucleated ooplastoid comprises less than the cytoplasmic volume of the original egg from which it is derived.

29. The stem cells of claim 19, wherein said enucleated ooplastoid comprises from about 10% to about 100% of the cytoplasmic volume of the original egg from which it is derived.



30. A stem cell which is produced by the method of (i) contacting a desired somatic cell or somatic cell nucleus with an ooplastoid, wherein said ooplastoid is derived from an enucleated oocyte; (ii) combining said somatic cell or somatic cell nucleus with said ooplastoid to create a nascent cell, and (iii) culturing said nascent cell to obtain pluripotent non-embryonic stem cells.

31. The stem cells of claim 30, wherein said cells are negative for expression of the SSEA-1 marker.

32. The stem cells of claim 30, wherein said cells express elevated alkaline phosphatase activity.

33. The stem cells of claim 30, wherein said cells are positive for expression of the TRA-1-81 marker and the TRA-1-60 marker.

34. The stem cells of claim 30, wherein said cells are positive for expression of the CCA-3 and CCA-4 Markers.

35. The stem cells of claim 30, wherein said cells differentiate into cells derived from mesoderm, endoderm and ectoderm germ layers when the cells are injected into a SCID mouse.

36. The stem cells of claim 30, wherein said cells are human.

37. The stem cells of claim 30, wherein said cells are non-human animal cells selected from the group consisting of dog, cat, mouse, rat, cow, pig, sheep, goat, horse, buffalo, llama, ferret, guinea pig and rabbit.

38. The stem cells of claim 30, wherein the nuclear DNA has been genetically modified.

39. The stem cells of claim 30, wherein said enucleated ooplastoid comprises less than the cytoplasmic volume of the original egg from which it is derived.

40. The stem cells of claim 30, wherein said enucleated ooplastoid comprises from about 10% to about 100% of the cytoplasmic volume of the original egg from which it is derived.

41. A nascent cell produced from the combination of a somatic cell nucleus and an enucleated zona pellucida free ooplastoid.

42. The nascent cell of claim 41, which is activated by a series of electrical pulses.

43. The nascent cell of claim 41, which is activated by the addition of a chemical activator.

44. The nascent cell of claim 41, which is activated by the addition of a chemical activator selected from the group consisting of ethanol, inositol trisphosphate, calcium ionophores, strontium ions, 6-dimethylaminopurine, cyclohexamide, and phorbol 12-myristate 13-acetate.

45. The nascent cell of claim 41, having from about 10% to about 100% of the cytoplasmic volume of the original egg from which it is derived.

46. The nascent cell of claim 41, having less than 50% of the cytoplasmic volume of the original egg from which it is derived.

47. A method of producing pluripotent, non-embryonic stem cells comprising the following steps:

(i) contacting a desired somatic cell or somatic cell nucleus with an ooplastoid, wherein said ooplastoid is derived from an enucleated oocyte;

(ii) combining said somatic cell or somatic cell nucleus with said ooplastoid to create a nascent cell;

(iii) activating said nascent cell; and

(iv) culturing said nascent cell to obtain pluripotent non-embryonic stem cells.

48. The method according to claim 47, wherein said somatic cell or somatic cell nucleus is a mature cell.

49. The method according to claim 47, wherein said somatic cell is an epithelial cell, lymphocyte or fibroblast.

50. The method according to claim 47, wherein said combining step involves intracytoplasmic injection of the somatic cell nucleus into the zona free reduced volume ooplastoid.

5 51. The method according to claim 47, wherein said combining step involves fusion in an electric field via electroporation.

52. The method according to claim 47, wherein said combining step involves fusion induced by electrodes that are introduced directly into the culture dish and electrical pulses  
10 administered to the couplets immediately following micromanipulation.

53. The method according to claim 47, wherein said combining step involves fusion in a fusion chamber.

15 54. The method according to claim 47, wherein said ooplastoid contains less than 50% of the cytoplasmic volume of a mature oocyte.

55. The method according to claim 47, wherein said ooplastoid contains from about 10% to about 100% of the cytoplasmic volume of a mature oocyte.

20 56. A cell line obtained according to the method of claim 47.

57. A method of producing pluripotent non-embryonic stem cells comprising the following steps:

- 25 (i) contacting one or more desired somatic cells or somatic cell nuclei with a super-ooplast derived from one or more enucleated zona pellucida free oocytes;  
(ii) dividing said super-ooplast into single nucleus containing nascent cells;  
(iii) activating said nascent cells; and  
(iv) culturing said nascent cells to obtain pluripotent non-embryonic stem cells.

30 58. The method according to claim 57, wherein said enucleated zona pellucida free super-ooplast comprises more than 100% of the cytoplasmic volume of a single egg.

59. The method according to claim 57, wherein said somatic cell or somatic cell  
35 nucleus is a mature cell.

60. The method according to claim 57, wherein said somatic cell is an epithelial cell, lymphocyte or fibroblast.

61. The method according to claim 57, wherein said dividing step involves  
5 partitioning said super-ooplast into separate single nuclei containing nascent cells.

62. The method according to claim 57, wherein said contacting step involves intracytoplasmic injection of said somatic cell nucleus into said super-ooplast.

10 63. The method according to claim 57, wherein said activation step involves fusion in an electric field via electroporation.

64. The method according to claim 57, wherein said activation step involves fusion in a fusion chamber.

15 65. The method according to claim 57, wherein said activation step involves fusion induced by electrodes that are introduced directly into the culture dish and electrical pulses administered to the couplets immediately following micromanipulation.

20 66. The method according to claim 57, wherein said nascent cell is activated using electrical pulses.

67. The method according to claim 57, wherein said nascent cell is activated during a fusion process.

25 68. A cell line obtained according to the method of claim 57.

69. A method of producing an ooplastoid comprising the following steps:  
30 (i) harvesting an oocyte from a female;  
(ii) maturing said oocyte to metaphase II;  
(iii) breaching or removing the zona pelucida of said metaphase II oocyte;  
(iv) enucleating said oocyte by removing the polar body and nuclear DNA of  
said oocyte through the breach of the zona pelucida or by oocyte partitioning; and  
(v) aspirating and pinching off an ooplastoid from said enucleated oocyte.

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70. The method of claim 69, wherein said oocyte is from a human.

71. The method of claim 69, wherein said oocyte is from a non-human animal  
selected from the group consisting of dog, cat, mouse, rat, cow, pig, sheep, goat, horse, buffalo,  
5 llama, ferret, guinea pig and rabbit.

72. The method of claim 69, wherein said zona pelucida is breached or removed using  
a chemical agent.

10 73. The method of claim 69, wherein said zona pelucida is breached or removed using  
mechanical action.

74. The method of claim 69, wherein said ooplastoid has from about 10% to about  
100% of the volume from the original oocyte.

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75. The method of claim 69, wherein said ooplastoid has from about 15% to about  
49% of the volume from the original oocyte.

76. The method of claim 69, wherein said ooplastoid has from about 17% to about  
20 33% of the volume from the original oocyte.

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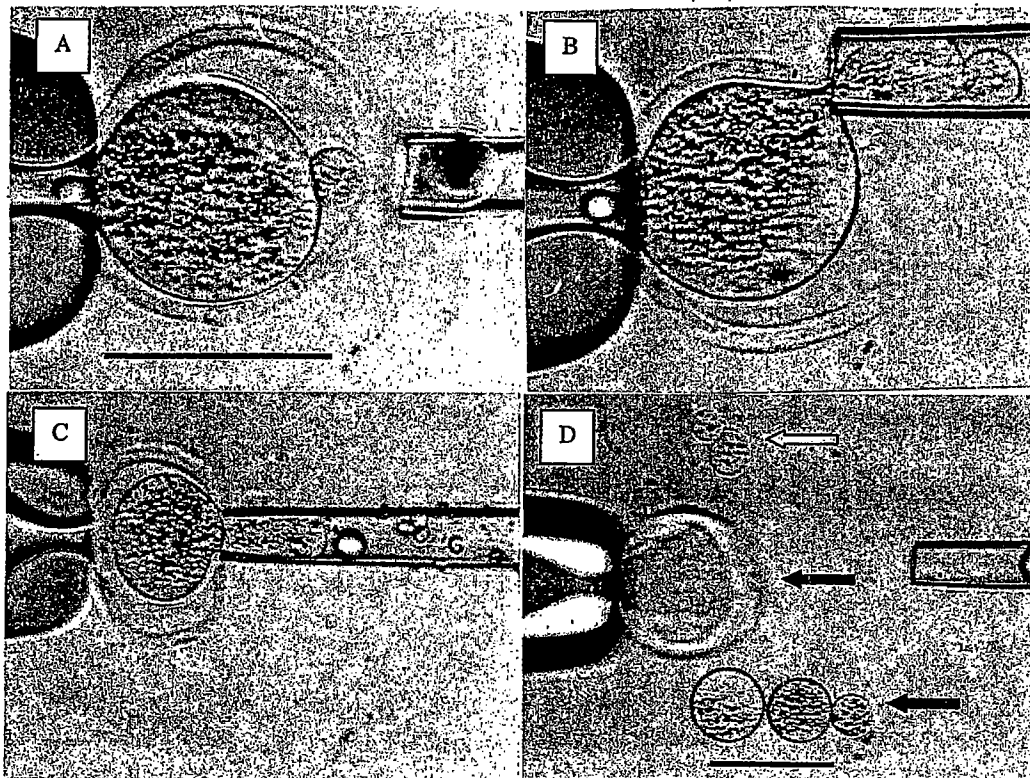
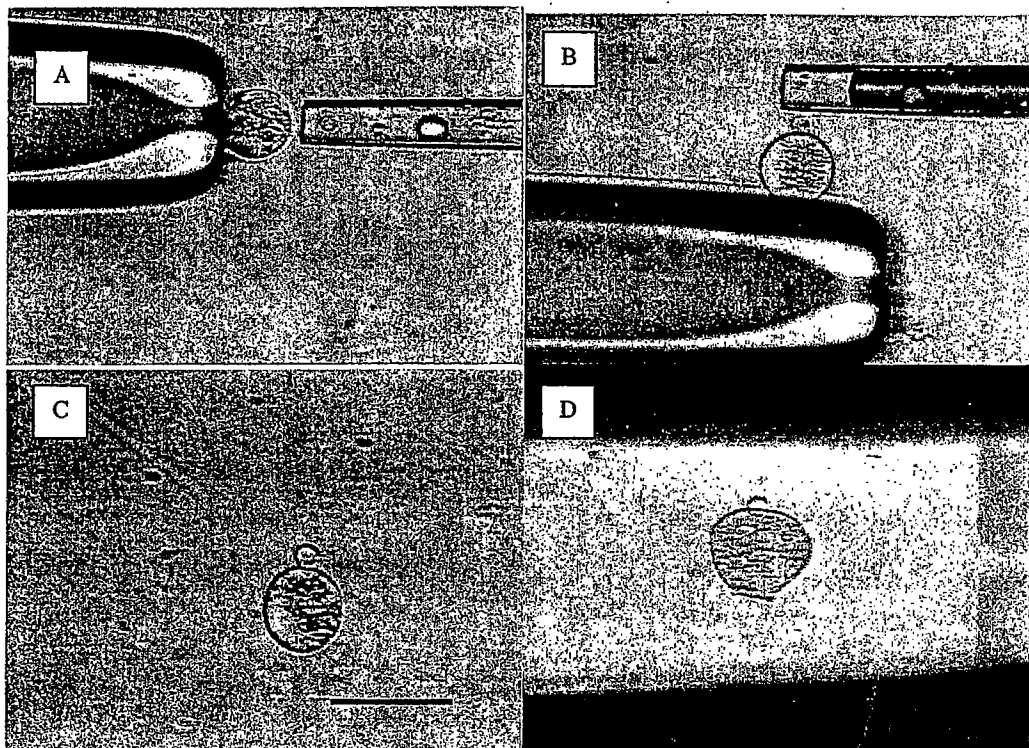
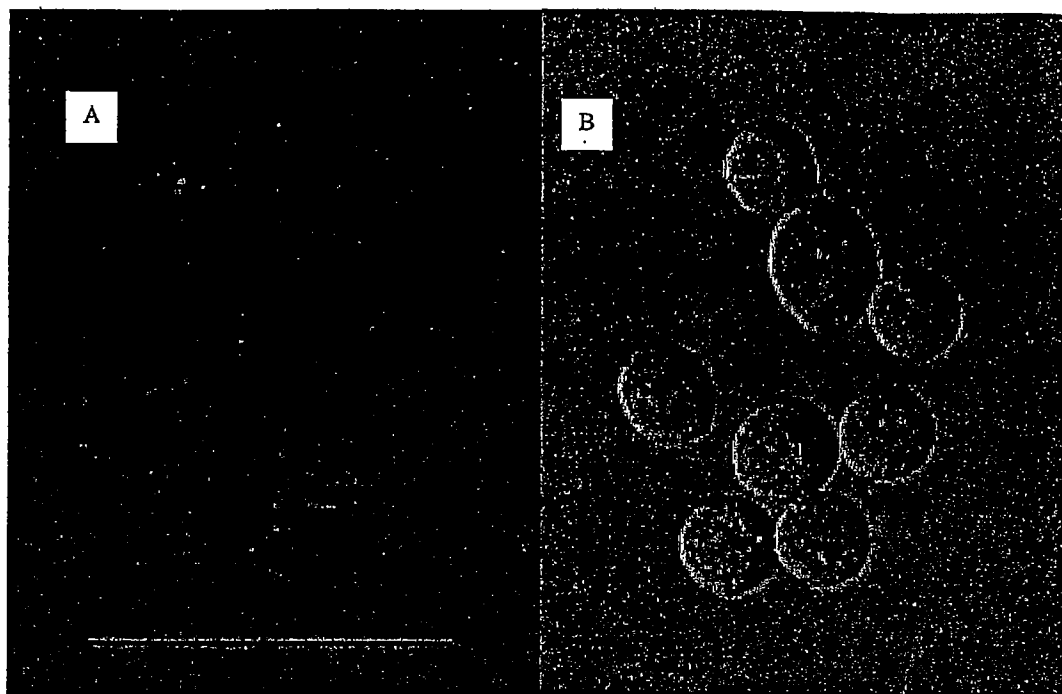


Figure 1

**Figure 2**



**Figure 3**